

11-1-2016

The Effects of Saxagliptin vs. Gliclazide and Taurine vs. Placebo on Endothelial Progenitor Cells and Arterial Stiffness in Type 2 Diabetes

Wan Aizad Wan Mahmood

Royal College of Surgeons in Ireland, wanmahmw@gmail.com

Citation

Wan Mahmood, WA. The Effects of Saxagliptin vs. Gliclazide and Taurine vs. Placebo on Endothelial Progenitor Cells and Arterial Stiffness in Type 2 Diabetes [MD Thesis]. Dublin: Royal College of Surgeons in Ireland; 2016.

This Thesis is brought to you for free and open access by the Theses and Dissertations at e-publications@RCSI. It has been accepted for inclusion in MD theses by an authorized administrator of e-publications@RCSI. For more information, please contact epubs@rcsi.ie.

— Use Licence —

Creative Commons Licence:



This work is licensed under a [Creative Commons Attribution-Noncommercial-Share Alike 4.0 License](https://creativecommons.org/licenses/by-nc-sa/4.0/).



**The Effects of Saxagliptin vs. Gliclazide and Taurine vs. Placebo on
Endothelial Progenitor Cells and Arterial Stiffness in Type 2
Diabetes**

Wan Aizad Wan Mahmood, MB BCh BAO MRCP (Ireland)

**Department of Endocrinology and Diabetes, Royal College of
Surgeons in Ireland, Connolly Hospital Blanchardstown, Dublin 15
Department of Medicine, Royal College of Surgeons in Ireland,
Education and Research Centre, Beaumont Hospital, Dublin 9**

**A thesis submitted to the School of Postgraduate Studies, Faculty of
Medicine and Health Sciences, Royal College of Surgeons in Ireland,
in fulfillment of the degree of Doctor of Medicine (MD)**

Supervisor: Dr. J.H.McDermott

October 2016

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD Doctor of Medicine is my own personal effort. Where any of content presented in the result of input or data from a collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work.

Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed _____

Student Number _____

Date _____

TABLE OF CONTENT

ABBREVIATIONS	7
LIST OF FIGURES	10
LIST OF TABLES	12
ABSTRACT STUDY 1	13
CARDIOVASCULAR SAFETY OF DPP-4 INHIBITION IN PARTICIPANTS WITH TYPE 2 DIABETES MELLITUS: ENDOTHELIAL PROGENITOR CELLS AS AN EARLY MARKER OF LONG-TERM CARDIOVASCULAR RISK	13
ABSTRACT STUDY 2	14
THE EFFECT OF TAURINE ON ENDOTHELIAL DYSFUNCTION IN TYPE 2 DIABETES MELLITUS: A PILOT STUDY	14
ACKNOWLEDGEMENT	15
1 INTRODUCTION	16
1.1 ENDOTHELIAL PROGENITOR CELLS	16
1.1.1 DISCOVERY OF ENDOTHELIAL PROGENITOR CELLS (EPCs)	16
1.1.2 MULTIPLE ORIGIN OF EPC	17
1.1.3 EPC CULTURE	19
1.1.3.1 Summary	21
1.1.4 ENDOTHELIAL PROGENITOR CELLS AND ANGIOGENESIS	22
1.1.5 EPC AND CARDIOVASCULAR DISEASE (CVD): CLINICAL STUDIES	25
1.1.5.1 Summary	27
1.1.6 FACTORS AFFECTING EPCs	28
1.1.7 EFFECTS OF STATIN ON EPC	29
1.1.8 EFFECT OF MULTIFACTORIAL TREATMENT ON EPC	30
1.1.9 SUMMARY	30
1.2 TYPE 2 DIABETES (T2DM)	32
1.2.1 DEFINITION, EPIDEMIOLOGY AND DIAGNOSIS OF T2DM	32
1.2.2 T2DM AND CARDIOVASCULAR DISEASE (CVD)	32
1.2.3 RELATIONSHIP BETWEEN TREATMENT OF T2DM AND CVD MORTALITY	33

1.2.4	GENERAL MANAGEMENT IN T2DM	34
1.2.5	METFORMIN AND CVD	35
1.2.6	SULPHONYLUREA AND CVD	36
1.2.7	THIAZOLIDINEDIONES AND CVD	39
1.2.8	DIPEPTIDYL-PEPTIDASE-4 INHIBITORS (DPP4i) AND CVD	42
1.2.9	ENDOTHELIAL DYSFUNCTION IN T2DM	44
1.2.10	EPC IN T2DM	47
1.2.11	EFFECT OF ANTI-DIABETIC TREATMENT ON EPC	50
1.2.11.1	Sitagliptin and EPC	50
1.2.11.2	Saxagliptin and Proangiogenic Cells (PACs)	51
1.2.11.3	Metformin, Gliclazide and EPC	52
1.2.11.4	Pioglitazone and EPC	53
1.2.12	SUMMARY	53
1.3	ARTERIAL STIFFNESS	54
1.3.1	ASSESSMENT OF ARTERIAL STIFFNESS	54
1.3.2	ARTERIAL STIFFNESS, PWV AND T2DM: CLINICAL STUDIES	57
1.3.3	EPC AND PWV IN DIABETES PATIENTS	59
1.3.4	SUMMARY	60
1.4	TAURINE	61
1.4.1	DISCOVERY AND SIGNIFICANCE OF TAURINE	61
1.4.2	TAURINE, DIABETES AND ENDOTHELIAL DYSFUNCTION	64
1.4.2.1	Taurine and Animal Models of Diabetes Studies	64
1.4.2.2	Taurine and Diabetes Human Studies	65
1.4.3	SUMMARY	67
2	<u>STUDY 1: CARDIOVASCULAR SAFETY OF DPP-4 INHIBITION IN PARTICIPANTS WITH TYPE 2 DIABETES MELLITUS: ENDOTHELIAL PROGENITOR CELLS AS AN EARLY MARKER OF LONG-TERM CARDIOVASCULAR RISK</u>	68
2.1	INTRODUCTION	68
2.2	METHODS	69
2.2.1	EPC ISOLATION AND ADHESION ASSAY	71
2.2.1.1	EPC Isolation	71
2.2.2	IDENTIFICATION OF EPC	72

2.2.3	COUNTING EPC	73
2.2.4	ADHESION ASSAY	74
2.3	STATISTICAL ANALYSIS	76
2.4	RESULTS	76
2.5	DISCUSSION	90
2.5.1	COMPARISON TO OTHER PUBLISHED STUDIES	90
2.5.2	DISCUSSION OF LITERATURE IN RELATION TO STUDY RESULTS	92
2.5.3	LIMITATIONS AND STRENGTHS	94
2.5.4	SUMMARY	95
3	<u>STUDY 2: THE EFFECT OF TAURINE ON ENDOTHELIAL DYSFUNCTION IN TYPE 2 DIABETES MELLITUS: A PILOT STUDY</u>	96
3.1	INTRODUCTION	96
3.2	METHODS	98
3.3	STATISTICAL ANALYSIS	101
3.4	RESULTS	101
3.5	DISCUSSION	107
3.5.1	COMPARISON WITH T1DM STUDY	107
3.5.2	DISCUSSION OF LITERATURE IN RELATION TO STUDY RESULTS	108
3.5.3	LIMITATIONS AND STRENGTHS	110
3.5.4	SUMMARY	111
4	<u>APPENDIX</u>	112
4.1	STUDY 1	112
4.1.1	PATIENT INFORMATION LEAFLET	112
4.1.2	CONSENT FORM	115
4.1.3	POSTER PUBLICATION	118
4.2	STUDY 2	119
4.2.1	PATIENT INFORMATION LEAFLET	119
4.2.2	CONSENT FORM	123
4.2.3	ETHICS COMMITTEE APPROVAL CERTIFICATE	126
4.2.4	TAURINE AND PLACEBO DOCUMENTATION	128
5	<u>REFERENCES:</u>	130

Abbreviations

ACEI	Angiotensin Converting Enzyme Inhibitor
ADA	American Diabetes Association
Ach	Acetylcholine
acLDL	Acetylated Low Density Lipoprotein
AGE	Advanced Glycation End Product
AMPK	AMP Activated Protein Kinase
ARB	Angiotensin Receptor Blocker
baPWV	Brachial Ankle Pulse Wave Velocity
bFGF	Basic Fibroblast Growth Factor
CAD	Coronary Artery Disease
CCF	Congestive Cardiac Failure
cfPWV	Carotid Femoral Pulse Wave Velocity
CFU	Colony Forming Unit
CFU-EC	Colony Forming Unit Endothelial Cells
CRP	C-Reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
CXCR	Chemokine Receptor
DBP	Diastolic Blood Pressure
DiL-LDL	Dil Labelled LDL
DPP4i	Dipeptidyl-Peptidase 4 Inhibitor
EASD	European Association for The Study of Diabetes
EC	Endothelial like cells
ECFC	Endothelial Colony Forming Cells
ecNOS	Endothelial Constitutive Nitric Oxide Synthase
EPC	Endothelial Progenitor Cells
EPO	Erythropoietin
FDA	Food Drug Administration
Flk	Fetal Liver Kinase
FMBRA	Flow Mediated Brachial Artery Reactivity

FMD	Flow-Mediated Dilatation
G-CSF	Granulocyte - Colony Stimulating Factor
GLP1a	Glucagon Like Peptide-1 Agonist
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HPMC	Hydroxypropyl Methylcellulose
HUVEC	Human Umbilical Vein Endothelila Cell
ICAM	Intercellular Adhesion Molecule
IGF1	Insulin Like Growth Factor 1
ITGB	Integrin Subunit Beta
KDR	Kinase Insert Domain Receptor
LDL	Low Density Lipoprotein
MCP	Monocyte Chemoattractant Protein
MetS	Metabolic Syndrome
MMP	Matrix Metalloproteinase
MNCs	Mononuclear cells
MP	Microparticles
NAC	N-Acetylcysteine
NO	Nitric Oxide
PAC	Proangiogenic Cells
PWV	Pulse Wave Velocity
RCT	Randomised Control Trial
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SCGF	Stem Cell Growth Factor
SDF	Stromal Cell-Derived Factor
SGLT2	Sodium-glucose co-transporter 2
STZ	Streptozocin
SU	Sulphonylurea
T1DM	Type 1 Diabetes

T2DM	Type 2 Diabetes
TG	Triglycerides
TNF	Tumour Necrosis Factor
TZD	Thiazolidinediones
UKPDS	United Kingdom Prospective Diabetes Study
VCAM	Vascular Adhesion Molecule
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor

List of Figures

Figure	Title	Page
Figure 1-1	Origin and differentiation of Endothelial Progenitor Cells	18
Figure 1-2	Mechanisms of EPC homing and differentiation	23
Figure 1-3	Progression of insulin resistance to T2DM parallels the progression of endothelial dysfunction to atherosclerosis	45
Figure 1-4	Measurement of carotid femoral PWV	55
Figure 1-5	The beneficial action of Taurine against hyperglycaemia induced endothelial dysfunction	62
Figure 2-1	Dual staining of EPC at day 7 under light microscopy (400X)	72
Figure 2-2	Dual staining of EPCs (DiL-AcLDL)	73
Figure 2-3	Dual staining of EPCs (<i>Ulex</i> Lectin)	73
Figure 2-4	EPC adhesion assay	75
Figure 2-5	Spearman correlation between diabetes duration and EPC numbers at baseline (Study 1)	79
Figure 2-6	Spearman correlation between diabetes duration and EPC adhesion at baseline (Study 1)	80
Figure 2-7	Spearman correlation between DBP and EPC adhesion (Study 1)	80
Figure 2-8	Changes in mean fasting glucose at 6 months compared to baseline according to treatment (Study 1)	87
Figure 2-9	Changes in mean BMI at 6 months compared to baseline according to treatment (Study 1)	87
Figure 2-10	Changes in median EPC numbers per hpf at 6 months compared to baseline according to treatment (Study 1)	88
Figure 2-11	Changes in median EPC adhesion at 6 months compared to baseline according to treatment (Study 1)	89

Figure	Title	Page
Figure 3-1	Comparing numbers of EPC/hpf based on treatment group (Study 2)	105
Figure 3-2	Comparing diastolic blood pressure (DBP) based on treatment group (Study 2)	106

List of Tables

Table	Title	Page
Table 1-1	Distribution of PWV values (m/s) in the reference value population (11 092 subjects) according to age and blood pressure category.	57
Table 2-1	Parameters at baseline (Study 1)	77
Table 2-2	Comparison between baseline parameters based on treatment (Study 1)	82
Table 2-3	Comparison between changes in parameters with treatment (Study 1)	85
Table 3-1	Parameters at baseline (Study 2)	102
Table 3-2	Comparison between changes in parameters at baseline, after Taurine and placebo supplementation (Study 2)	104

Abstract Study 1

Cardiovascular safety of DPP-4 inhibition in participants with Type 2 Diabetes Mellitus: Endothelial Progenitor Cells as an early marker of long-term cardiovascular risk

Endothelial Progenitor Cells (EPCs) play a central role in cardiovascular repair and increasing EPC number is a strong determinant of future cardiovascular risk(1). Interventions, which modify cardiovascular risk, have early effects on EPC number and function (2). Establishing cardiovascular safety of any new anti-diabetic agent typically requires long-term post-marketing trials. DPP-4 inhibitors are a new class of glucose-lowering medication(3). We aimed to evaluate the long-term cardiovascular safety of Saxagliptin (SAX, a DPP-4 inhibitor) versus Gliclazide Modified Release (GLC) using EPC number and function as surrogate markers of cardiovascular risk. 18 participants with T2DM on Metformin monotherapy were randomized to SAX (n=7) or GLC (n=11). EPC number and adhesion capacity were measured, using methods as previously described (4), at baseline and at 6 months after treatment. There was no difference in the median EPC number of 33 (19 – 44) vs. 25 (22 – 46) cells per high power field (HPF), $p=0.9$ or adhesion capacity of 0.22 (0.17 – 0.86) vs. 0.27 (0.1 – 0.67) fluorescence units, $p=0.75$ at baseline between SAX and GLC groups respectively. Compared to baseline there was no change in median EPC number [SAX: 35 (28-38) vs 33 (19-44) cells/HPF, $p=0.9$ and GLC: 39 (28-46) vs 25 (22-46) cells/HPF, $p=0.24$] or adhesion capacity [SAX: 0.3 (0.17 – 0.65) vs 0.22(0.17 – 0.86), $p=1.0$, GLC: 0.32 (0.14 – 0.84) vs 0.27 (0.1 – 0.67) fluorescence units, $p=0.88$] after 6 months treatment in either group. In summary, we found no difference in EPC number or function in participants treated with SAX versus GLC for 6 months. These results may suggest a similar cardiovascular safety profile of SAX to GLC, a well-established treatment for T2DM.

Abstract Study 2

The Effect of Taurine on Endothelial Dysfunction In Type 2 Diabetes Mellitus: A Pilot Study

There is a two- to three-fold increase in cardiovascular (CVD) mortality in T2DM patients compared to aged-matched non-diabetic subjects(5). Endothelial dysfunction characterized by arterial stiffness predicts this CVD risk(6). Endothelial Progenitor Cells (EPCs) are bone marrow derived cells responsible for cardiovascular repair, act as a surrogate biological marker for vascular dysfunction, and predict cumulative future cardiovascular risk(7). Taurine is a sulfur-containing amino acid, synthesized in the body in limited amounts, and is present mainly in seafood(8, 9) which has been shown to have a beneficial effect in hypertension(10, 11), hypercholesterolaemia(12, 13), and endothelial function (14, 15). Taurine supplementation for 2 weeks improves arterial stiffness in patients with T1DM(16). We carried out a pilot study examining the effects of taurine on 11 T2DM patients in a randomized, double blind, cross-over placebo design. We evaluated any changes to EPC numbers and function. We also compared the degree of arterial stiffness using PWV. There was a trend towards an improvement in the number of EPC/hpf following taurine (40 ± 7 cells) and placebo (37 ± 10 cells) when compared to baseline (32 ± 8 cells, $p=0.08$). Similarly, there was a trend towards improvement in DBP following taurine (78.6 ± 7.4 mmHg) and placebo (79.3 ± 7.5 mmHg) when compared to baseline (86.3 ± 10.2 mmHg, $p=0.08$). There were no differences in the EPC adhesion capacity between baseline ($n=10$, 0.66 ± 0.4 fluorescence unit), taurine (10 , 0.97 ± 0.8 fluorescence unit) and placebo (11 , 0.94 ± 0.5 , $p=0.43$ fluorescence unit) group. There were also no differences in the measurements of PWV baseline [8.7 (8.0 - 8.4) m/s], taurine [9.1 (8.3 - 9.6) m/s] and placebo [8.9 (8.8 - 9.1) m/s, $p=1.0$]. These results may be interpreted as an absence of benefit of Taurine in modulating known markers of endothelial dysfunction in T2DM. The downward trend in DBP and an upward trend in the numbers of EPC seen in this pilot study, however, will require further evaluation in a larger cohort.

Acknowledgement

First and foremost, I would like to sincerely thank my supervisor Dr John McDermott for the opportunity to do this research in your department. I am eternally grateful for your guidance throughout the research, thesis writing and throughout my career to date.

I would also like to thank Prof Seamus Sreenan for your support and guidance over the last number of years. Thank you for your trust and for giving me the opportunity to train in the field of Endocrinology and Diabetes.

A special thank you to our Diabetes Specialist Nurse, Sandie McAteer; for your assistance with the blood work and for being an integral part during the intervention study.

Thank you to Emer Reeves, Lance Hudson, Irene Oglesby, Tomas Carroll and David Bergin for your assistance in the laboratory work.

I would like to express my gratitude to all the patients who took part in the research studies.

Finally I would like to thank my family and friends for their support and love.

To my lovely wife, Chai, I dedicate this thesis to you. Thank you for your love, your support, encouragement and your patience throughout this journey of ours. I'm glad to have met you; you are my 'rock' and without you, none of this would have been possible.

1 Introduction

1.1 Endothelial Progenitor Cells

1.1.1 Discovery of Endothelial Progenitor Cells (EPCs)

Endothelial Progenitor Cells (EPCs) are circulating bone marrow derived cells capable of postnatal vasculogenesis and vascular homeostasis (17). The EPC was first described by Asahara et al who demonstrated that circulating $CD34^{+}$ cells are able to differentiate into endothelial-like cells ex-vivo(18).

These haematopoietic $CD34^{+}$ mononuclear cells ($MB^{CD34^{+}}$) were isolated from peripheral blood using magnetic beads, and seeded on fibronectin plates. Spindle shaped cells developed after 3 days incubation and the numbers of these attached cells increased with time. These attached cells ($AT^{CD34^{+}}$) proceeded to develop cellular networks and tube-like structures.

These $AT^{CD34^{+}}$ cells were also shown to express endothelial constitutive nitric oxide synthase (ecNOS), Fetal Liver Kinase 1/Kinase insert Domain Receptor (Flk-1/KDR), TIE2 and E-Selectin which are markers of endothelial cell phenotype.

To prove that these cells are involved in vessel regeneration, Asahara used a mouse and rabbit model of hind-limb ischaemia. Infused $MB^{CD34^{+}}$ cells were shown to migrate to foci of angiogenesis, proliferate, integrate into the capillary vessel wall, and contribute to neoangiogenesis in the ischaemic hind limb of the mouse and rabbit.

Similar to Asahara's work, Shi et al(19) demonstrated that a subset of $CD34^{+}$ haematopoietic stem cells were able to differentiate into endothelial cell lineages. These cells were also expressing endothelial proteins such as von Willebrand factor (vWF) and were able to incorporate Dil-labeled LDL (Dil-LDL). In an in-vivo model, Shi et al transplanted genetically tagged bone marrow cells from a donor canine. A Dacron graft, impermeable to capillary ingrowth from the surrounding perigraft tissue, was implanted in the descending thoracic aorta of the

recipient animal. Cells with endothelial morphology were identified on the graft and only donor alleles were detected in DNA of these cells.

These studies, taken together strongly suggest that a subset of CD34⁺ cells from the bone marrow can be mobilized into peripheral circulation and colonize endothelial surfaces. The studies challenge the original understanding of postnatal vasculogenesis that postnatal angiogenesis only occurred through proliferation, migration and remodeling of pre-existing endothelial cells (20-22).

1.1.2 Multiple Origin of EPC

There are two main methods to isolate EPC. Firstly, by identifying cell surface progenitor cell markers such as CD34, CD 133 and endothelial marker VEGFR2. Secondly, by using differential cell cultures technique, together with growth medium on different types of adhesion surface(23, 24)

CD34 expressivity however, is not exclusively seen on haematopoietic stem cells, but also on mature endothelial cells(25)(Figure 1-1). The more immature haematopoietic stem cell with marker CD133 could also differentiate to endothelial cells in vitro(26). Using vascular endothelial growth factor (VEGF), which is a major inducer of vasculogenesis, stem cell growth factor (SCGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) in culture media, these AC133⁺ cells were able to differentiate into endothelial-like cells (EC) in-vitro. The adherent EC displayed endothelial characteristics including expression of CD34, CD31, vascular endothelial cadherin (VE-Cadherin), KDR, Tie-2, Ulex europeus-agglutinin-1 and vWF respectively, and the presence of Weibel-Palade bodies(26).

Monocytic cells, specifically the CD14⁺/CD34⁻ cells may also differentiate into endothelial-like cells. Under angiogenic stimulation with VEGF, basic

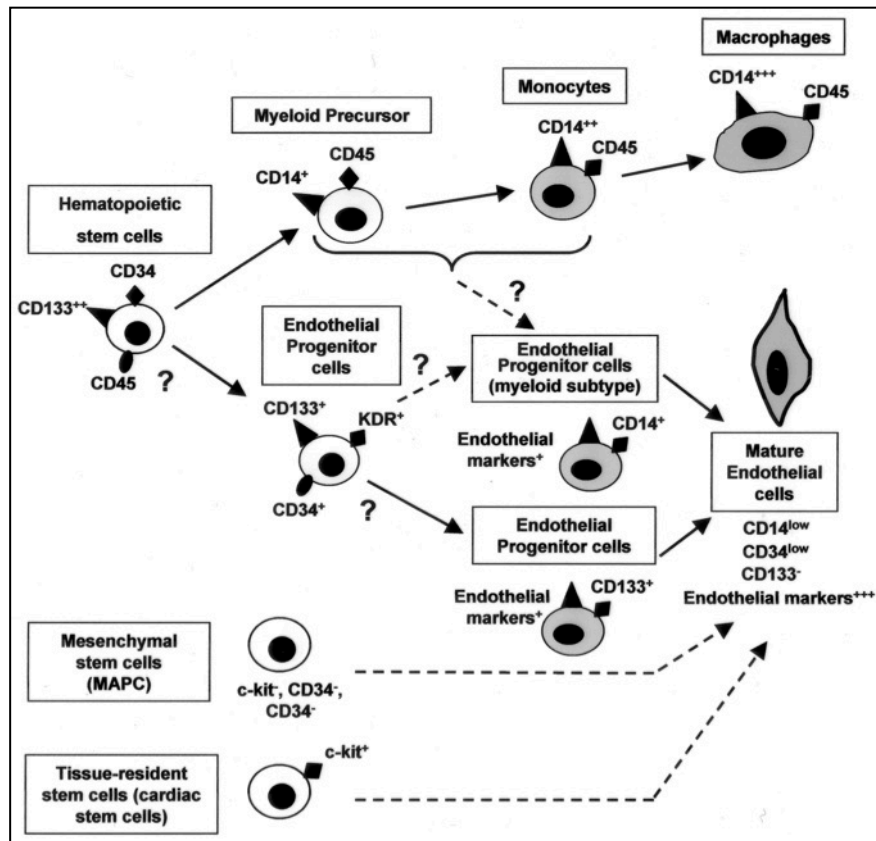


Figure 1-1 Origin and differentiation of Endothelial Progenitor Cells. Scheme depicts the potential origin and differentiation of EPC from haematopoietic stem cells and non-haematopoietic cells. (Adapted from Urbich et al. Circ. Res. 2004; 95; 343-353)

fibroblast growth factors (bFGF) and insulin like growth factors (IGF-1), these cells were induced to express endothelial markers (vWF, VE-cadherin and ec-NOS) and displayed similar morphology to the original description by Asahara et al. In the same study, CD34⁺ cells, under similar culture medium, demonstrated similar cell morphology, albeit with a smaller percentage compared to CD34⁻ cells(27). The exact origin of monocyte-derived EPC remained to be elucidated as fresh monocyte also displayed typical endothelial cells characteristics (LDL-uptake, Lectin binding, expression of CD31, CD 105 and CD 144) making them indistinguishable to monocyte-derived EPC(28). Mice study however, did demonstrate equal improvement in neovascularization of hind-limb ischaemia from EPC derived from clonal expansion of either monocytic CD14⁺ or non-monocytic CD14⁻ (29).

To further clarify the antigenic profile of EPC, a proteomic method was used to identify microparticles (MP) on EPC. As MPs retain membrane antigen specific from the parent cells, this made them ideal candidate to clarify the cellular progeny of EPC culture. Prokopi et al demonstrated that in a conventional EPC isolation, MPs originating from platelets were taken up by the mononuclear cells population during culture. This contributes to the currently known “endothelial” characteristics (CD31, vWF, and lectin-binding). This novel finding challenges the previous preclinical and clinical studies describing EPC cellular progeny especially when unselected bone marrow mononuclear cells were used(30).

1.1.3 EPC Culture

Cell culture methods to isolate EPC have evolved significantly since the original description by Asahara et al(1, 7, 31-33). The main differences include culture duration, types of adhesion matrix molecule and utilizing a pre-plating procedure(24).

To isolate mononuclear cells (MNCs) from peripheral bloods, a Ficoll density gradient centrifugation is used, and these isolated MNCs, are cultured on matrix molecules such as fibronectin(2, 34). Adherence to fibronectin, with dual positivity staining to acetylated low-density lipoprotein (acLDL) and *Ulex* Lectin are typical characteristics of EPCs identified in-vitro by most groups(2, 4, 7, 35, 36). The short duration protocol such as used in our study, yielded cells with myeloid/haematopoietic characteristics. The ‘early EPCs’ (after 4 days cultured on fibronectin surface) are spindle shaped and express CD45, and typical myeloid markers CD14 and CD11b(24, 32).

In order to remove potential monocytes and macrophages, Hill et al pre-plated MNC and selected only non-adherent cells for cultures to form cluster of cells with spindle shapes sprouting at the periphery termed colony forming unit endothelial cells (CFU-EC)(7). Similar to ‘early EPCs, CFU-EC also expressed similar markers of myeloid and haematopoietic cells (24).

Long-term culture of 'early EPC' yielded more mature endothelial cell phenotype, often referred to as 'late' or 'outgrowing' EPC(24). Late EPC, in contrast, have a cobblestone shape; appear after 2 to 3 weeks of culture with exponential growth at 4 to 8 weeks, and the majority are derived from a CD14⁻ subpopulation(32). In a more differentiated state, these EPC will increase the expression of endothelial markers such as KDR, VE-cadherin, eNOS, caveolin-1, vWF, and CD62E(2, 37). KDR is generally a marker that signifies progenitor cell commitment to differentiate into endothelial cells, being expressed at more immature stage than CD31 and vWF(38). Late EPC are considered more capable of in-vitro tube formation compared to early EPC(29, 32).

Culturing on collagen however, for more than 14 days formed 'endothelial colony forming cells' (ECFC)(24, 33). These cells do not have the haematopoietic and myeloid markers, appeared to be clonally unrelated to CFU-ECs and are capable of forming vascular networks(24). Although ECFCs are considered to be the 'true' EPC, the exact origin of these cells remains to be elucidated as they lack the progenitor markers and are indistinguishable from the mature endothelial cells(24).

Some authors believe that acLDL uptake and lectin binding are no longer sufficient to characterize EPC and that a prolonged culture for 2 weeks without pre-plating is a valuable alternative compared to other methods(34). Many authors feel that a wider expression of endothelial markers (CD31, KDR, vWF and eNOS) together with typical endothelial cell morphology would define a minimum criterion for EPC identification; and that additional criteria should include identification of additional markers (CD45, CD34, CD133, CD 117) and further assay demonstrating ability of cells to incorporate into endothelium.

Our group isolates EPCs from peripheral blood MNC and culture on fibronectin plates for 7 days(4, 39). We discard the non-adherent cells and change endothelium media on Day 4. We confirm adherent cells as EPCs based on the uptake of acLDL and Lectin on Day 7 adherent cells. We also have performed Western blot analysis and laser scanning

cytometry to confirm the co-expression of haematopoietic and endothelial stem cell markers CD34 and VEGF on the adherent cells at day 7(4). Based on our culture technique and the morphology of cells during culture, it is likely that our EPC also originated from the myeloid precursors(24, 27). Our methods are well established (4, 34, 39) and it has been used by Choi et al, linking EPCs to coronary artery disease risk in patients with chronic renal failure compared to healthy volunteers(40).

1.1.3.1 Summary

Under correct growth factor stimulation, bone marrow derived pluripotent cells such as haematopoietic and myeloid stem cells are able to differentiate into EPC. Depending on the duration of culture, MNC will develop into 'early' and 'late' EPC with differing characteristics. There are some agreements regarding the minimum criteria for identification of EPC; however, in order to improve the specificity of EPC identification, there are still areas that will require further studies. These include further development in in-vitro colony assays, in-vivo tracing of endothelial lineage, further epigenetic studies to understand transfer of material, intercellular communications and haemato-endothelial transition studies(24).

Both flow cytometry and culture methods continue to evolve and these techniques are still used in various studies involving EPCs, particularly in the area of cardiovascular disease (CVD)(40), ischaemic stroke(35), pharmacology(41), diabetes and vascular(42) diseases.

1.1.4 Endothelial Progenitor Cells and Angiogenesis

Endothelial Progenitor Cells contribute to angiogenesis through improvement in neovascularization by influencing endothelial regeneration (25).

In injured or denuded arteries, EPCs have been shown to home to the injured site (43), induce rapid regeneration of the endothelial monolayer and further prevent restenosis by synthesizing anti-proliferative mediators such as nitric oxide (44, 45).

In tissue ischaemia, mediated by activation of hypoxia-sensing systems, such as hypoxia inducible factor (HIF)-1 α , there is up-regulation of VEGF and stromal cell-derived factor-1 α (SDF-1 α) by the injured tissue(46, 47). These substances activate the matrix metalloproteinase-9 (MMP-9)-dependent pathway resulting in release of progenitor cells from the bone marrow (48-51).

Mobilization of bone marrow derived EPCs is the first step following tissue ischaemia (25)(Figure 1-2). Other potent factors helping this process include granulocyte-colony stimulating factor (G-CSF), GM-CSF(51), and EPO(52, 53).

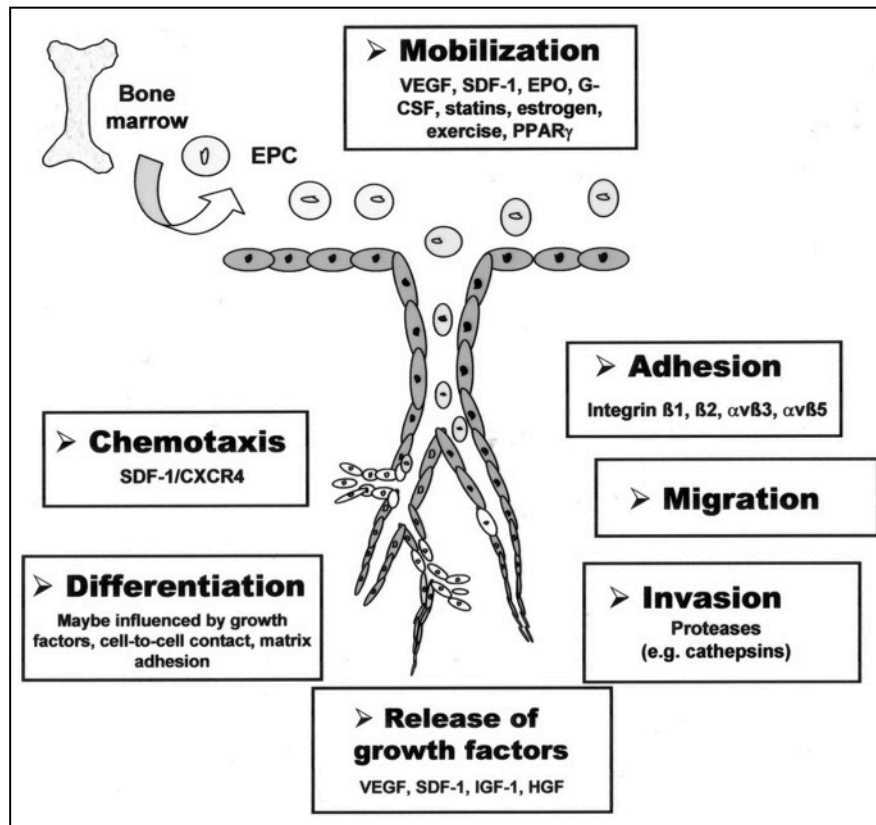


Figure 1-2 Mechanism of EPC homing and differentiation. Recruitment and incorporation of EPCs into ischemic tissue requires a coordinated multistep process including mobilization, chemoattraction, adhesion, transmigration, migration, tissue invasion, and in situ differentiation. (Adapted from Urbich et al. Circ. Res. 2004; 95; 343-353)

The intensity of the ischaemia or injury determines the EPC incorporation rate. This fact was demonstrated by various studies, which illustrated large differences in the percentage of endothelial markers expressed (48, 54-60) on new-formed vessels depending on the severity of the ischaemia. Some studies demonstrated a low incorporation rate (29, 61), which suggest that neovascularization does not depend solely on EPC incorporation, but also depends on the EPC's paracrine release of pro-angiogenic growth factors such as VEGF, Hepatocyte Growth Factor (HGF), and G-CSF (62). These growth factors may influence proliferation, migration and survival of mature endothelial cells(21).

Therapeutically, HMG-CoA reductase inhibitors (statins) have been shown to increase the proliferation and mobilization of EPC, and to prevent EPC senescence and apoptosis through phosphatidylinositol 3-kinase (PI3K/Akt pathway)(41, 59, 63, 64). Interestingly, this similar pathway is augmented by VEGF, EPO, estrogen and exercise suggesting potential sharing of a common pathway to induce EPC mobilization(25).

Once EPC are mobilized, they adhere to sites of injury or ischaemia. EPCs depend on integrin protein to adhere to ischaemic endothelial cells and extracellular matrix protein(25). In a study using volunteers EPC, fibronectin receptor (composed of $\alpha_5\beta_1$ integrin subunits) and α_v and β_5 integrin subunits were upregulated in response to a common lipid-lowering agent, statin. By blocking these receptors with cyclic RGD peptides in an animal model with carotid artery denudation, the incorporation of EPCs into the injured artery were significantly reduced, proving the functional relevance of these integrin to EPC adhesions(44).

SDF-1 α , which binds to a 7-transmembrane-spanning G protein-coupled chemokine receptor-4 (CXCR4), have been shown to play an important role in regulation of cellular function of EPCs such as mobilization from bone marrow, migration, proliferation, and survival (65-67). EPC migration is mediated by E- and P-selectins. Other molecules such as intracellular adhesion molecule-1/leukocyte function-associated antigen-1/vascular cell adhesion molecule-1/very late antigen (VLA)-4 ligand pairs and junctional adhesion molecules strengthen the adhesions with β_2 integrins mediates the transmigration of EPC thereafter.(68)

Little was known with regards to the downstream signal transduction pathway used by EPC for migration but over the last number of years, studies have identified the PI3K/Akt and MAPK/ERK pathways as potential signal transduction pathways for this purpose(41, 69-72). Indeed, many other cell types have been shown to require both pathways in facilitating migration capacity(69, 72-74). Wang et al(72) demonstrated that the PI3K/Akt and not the MAPK/ERK pathway was required for SDF-1 α –mediated migration of haematopoietic progenitor cells and primary

marrow CD34⁺. Floridi et al and Sotsios et al however, demonstrated that both pathways were involved(69, 71). By using PI3K inhibitors (LY294002 and wortmannin) and MEK inhibitor (PD98059), Zheng et al(75) indicates that PI3K/Akt and not MAPK/ERK activation is involved in the SDF-1 α /CXCR4-mediated EPC migration.

In an early animal model, Asahara et al, showed an increased EPC population and differentiation by nearly 5 and 4-fold respectively following hind-limb ischaemia(18). To investigate EPC kinetics during tissue ischaemia, mice with induced hind-limb ischaemia and sham-operated controls were used. These mice were subjected to cornea micro-pocket surgery. There was 1.3-fold increase in neovascularization of the cornea in the mouse with hind-limb ischaemia compared to mouse with non-ischaemic sham-operated control(51). Another study used culture-expanded EPC from human donors, which were successfully transplanted in an athymic mouse model with hind-limb ischaemia. The transplanted EPC promoted neovascularization of ischaemic tissue and reduced limb necrosis and autoamputation by 50%(31).

1.1.5 EPC and Cardiovascular Disease (CVD): Clinical Studies

The capabilities of EPC to home to sites of neovascularization and differentiate into endothelial cells contribute greatly to the modern understanding of vasculogenesis. The role of EPC in improving ischaemic-induced damage led to studies of EPC in influencing cardiovascular risk.

In 2001, Vasa et al looked at the relationship between atherosclerotic risk factors and the number and functional activity of EPCs(1). Compared to age-matched volunteers, participants (n=45) with angiographically documented coronary artery disease (CAD) had 40% lower EPC level. There was a negative correlation between the number of risk factors and EPC level. Smoking was an independent predictor of reduced EPC levels while no association was seen with age or low-density lipoprotein (LDL) cholesterol. Similarly, the migratory capacity of EPC was significantly impaired in CAD group with negative correlation seen with the total

numbers of risk factors for CAD. In multivariate analysis, hypertension was shown to be a significant risk factor for reduction in EPC migration. In summary, participants with CAD had lowered EPC levels and impairment of functional capacity, which correlated with numbers of risk factors for CAD such as smoking and hypertension.

What about in subjects without CAD who may or may not have risk factors for cardiovascular disease? Would similar risk factors affect EPC number and function prior to the development of CAD?

To answer this, Hill et al(7) examined 45 subjects without CAD. All were male, with and without risk factors for cardiovascular disease. Their risk factor burden was calculated using the Framingham risk score, which has been proven to predict the risk of CAD in disease-free subjects(76).

In this study, flow mediated brachial artery reactivity (FMBRA) also known as flow-mediated dilatation (FMD) was used to evaluate endothelial function. Endothelium-dependent vasodilatation was assessed by measuring the maximal increase in the diameter of brachial artery during reactive hyperaemia evoked by the release of cuff inflated to 255mmHg for 5 minutes on the upper arm, proximal to the measurement site. This is a well-recognized technique in the evaluation of endothelial function(77) and was used as a surrogate measurement of ongoing function of EPCs based on the risk burden.

In subjects without CAD, adjusted for age, hypercholesterolaemia was significantly associated with lower EPC colony forming unit (EPC CFU). Framingham score was negatively correlated; while FMBRA was positively correlated with EPC counts. At the highest reactivity of FMBRA, there was 3-fold increase in the number of EPCs. In the multivariate analysis, FMBRA was an independent predictor of EPC counts.

Interestingly, subjects with high and low EPC counts had preserved FMBRA or depressed FMBRA irrespective of the Framingham score. In multivariate analysis of FMBRA, when both Framingham score and

number of EPC inserted, EPC count was significant predictor over and above the effects of Framingham score.

1.1.5.1 Summary

Both studies(1, 7) demonstrated that EPCs from high-risk subjects were fewer and become senescent more rapidly compared to low-risk subjects. Although cause and effect were not established, the findings by Hill et al and Vasa et al suggest that there may be a link between prolonged exposure of cardiovascular risk factors, EPC physiology, endothelial function and coronary artery disease. Levels of EPC may act as a surrogate biological marker for vascular function and cumulative cardiovascular risk. Similarly, endothelial injury in the absence of sufficient circulating EPC may affect the progression of CVD(7).

The nature of both studies was not adequate to establish cause and effect. In order to establish the relationship between EPC, FMBRA and prediction of CVD, studies where EPCs are experimentally manipulated and the biological and therapeutic results analyzed, were needed

Schmidt-Lucke et al (78) were the first to demonstrate that a reduced level of EPC independently predicts future cardiovascular (CV) events in a prospective study. Subjects included participants with stable CAD (n=44), unstable CAD (n=33), acute coronary syndrome (ACS, n=43) and healthy control (n=43); with median follow up of 10 ± 12.1 (range 1-48) months. EPC were measured at inclusion. Subjects with CV events had lower EPC compared to subjects without CV events. Crude hazard ratio for CV events was 6.3 and after adjustment for disease activity and risk factor load for CAD, reduced EPC accounted for nearly 4-fold increased risk of suffering CV events during follow up. Thus, the number of circulating EPCs independently predicts atherosclerotic disease progression(78). Fadini et al quantified the level of CD34+ EPC in 214 subjects with different level of CV risk. There were negative correlations between the level of CD34+ cells with CV risks with a synergistic effect from each component of metabolic syndrome(38). Both studies support the role of EPC as a surrogate marker for CV risks.

1.1.6 Factors affecting EPCs

Several studies have examined factors potentially modulating the number of EPCs. Exhaustion of EPC pool in the bone marrow, reduced mobilization, reduced survival, and/or differentiations have been suggested as possible mechanisms for a reduction in circulating EPC number.

A study by Aicher et al used a mice model deficient in endothelial nitric oxide synthase (eNOS) and demonstrated that deficiency in eNOS reduced mobilization of EPCs due to reduction in vascular endothelial growth factor (VEGF) production(61).

Hill et al performed cell senescence analysis in a subset of participants with CVD risk factors. By using β -galactosidase as a marker of cell senescence, they demonstrated that subjects with high risk for CVD had higher percentages of EPCs with a senescence phenotype compared to low risk subjects(7). Presence of diabetes, obesity, hypertension, hyperlipidaemia, previous CVD and age above 50 years old are other factors associated with lower level of EPC as determined by the CD34⁺ level(38). There is an inverse correlation between the levels of EPCs and the numbers of cigarettes smoked. Smoking cessation increased EPC numbers while smoking resumption decreased EPC numbers towards the level pre-cessation(79).

Sata et al examined the capabilities of the hematopoietic stem cells to differentiate to endothelial cells. Differentiations of these bone marrow CD34⁺ cells to EPCs are dependent on the endothelial growth factors (e.g. VEGF)(80). This VEGF induced differentiation is further inhibited by oxidized LDL as demonstrated by Imanishi et al(81). When isolated human mononuclear cells (MNC) are incubated with VEGF and oxidized LDL, oxidized LDL affected the differentiation to EPC in a dose dependent manner through deactivation of Akt pathway. More importantly, this inhibitory effect induced by oxidized LDL was prevented by pretreatment with atorvastatin(81). Certainly, this and other studies

(41, 44, 59, 63, 64) provided the background for further work in the role of statin in improving EPC numbers and functions.

1.1.7 Effects of Statin on EPC

Higashi et al looked at the effect of Pravastatin on flow-mediated dilatation (FMD), circulating EPCs and EPC migration in subjects with low level of high-density lipoprotein (HDL) and no CV risk factors versus placebo control subjects. FMD improved after 4 weeks in treated group compared to placebo group. Changes in HDL, numbers and migration of EPC were independent predictors of augmentation of FMD with Pravastatin(82).

The effect of statins on EPC was also examined in participants with established CAD. Participants who started atorvastatin or had an increase in atorvastatin dose for 4 weeks had a reduction of endothelial cell (EC) apoptosis by 50% with a doubling of EPC numbers compared to participants who were given ezetimibe in addition to stable dose of atorvastatin therapy(83).

One study examined the effect of statins on EPC in participants with T2DM. A high risk CVD group of participants (n=26) including 19 T2DM participants were randomized to either Pitavastatin or Atorvastatin for 12 weeks. Both statins reduced plasma lipid levels while higher EPC levels were observed in the Pitavastatin group, suggesting possible differential effects of different statins on EPC(84).

More recently, a systematic review of randomized trials of statin therapy and EPC revealed varying percentage increases in EPC numbers with a median increase of 70.2%. Some of the main weaknesses in the studies reviewed included significant heterogeneity in patient population, statin type and the definition of EPC used(85). Nevertheless, there is no doubt that statin has significant benefit on EPC numbers and function and this fact contributes to our understanding of vascular remodeling and physiology.

1.1.8 Effect of Multifactorial Treatment on EPC

Reinhard et al evaluated the effect of a multifactorial treatment strategy on EPC numbers in subjects with T2DM (n=28). EPC culture was performed at baseline and at 30 and 90 days. Patients were started on multifactorial treatments covering aspects of glycaemic control, lipid and blood pressure lowering, antithrombotic therapy and lifestyle modification. Metformin, statin, aspirin and Angiotensin Receptor Blocker (ARB) were pharmacological agents used. EPC numbers were analyzed based on mono-, dual-, triple- or quadruple therapy used. There was a non-significant increase of EPC numbers by 15% after 30 days and a significant increase of 35% by 90 days of multifactorial treatment compared to baseline. EPC count was reduced by 32% in medically untreated patient (n=2), and increase by 63% in patients with quadruple therapy(86).

Treatment of hypertension with ARB (olmesartan and irbesartan) for 12 weeks has been shown to increase EPC numbers in T2DM patients independent of blood pressure reduction to the same magnitude as statins(87). Additionally, in normal healthy volunteers, treatment with low dose aspirin (equivalent of 75mg in-vivo) has an anti-senescence, pro-migratory and pro-adhesive effect on EPCs(88).

Anti-diabetic medications have been shown to have positive effects on EPC and this will be discussed in detail in the diabetes section.

1.1.9 Summary

EPC are bone marrow derived cells that challenge the original understanding of postnatal angiogenesis. EPC has multiple origins, and may be identified by the presence of specific cell markers CD34, CD133, and KDR, together with dual staining with acLDL and *Ulex* lectin. EPC improves neovascularization, thus contributes to angiogenesis and the number of circulating EPCs independently predicts atherosclerotic disease progression. Diabetes, obesity, hypertension, hyperlipidaemia, and smoking are among factors identified affecting EPC differentiation, and mobilization. Multidrug treatment, statin and antidiabetic treatment

especially Sitagliptin have been shown to improve EPC numbers and function.

1.2 Type 2 Diabetes (T2DM)

1.2.1 Definition, Epidemiology and Diagnosis of T2DM

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defect in insulin secretion, insulin action or both (89).

T2DM is characterized by a progressive insulin secretory defect on a background of insulin resistance. It is a complex chronic illness requiring continuous medical care with multifactorial risk reduction strategies beyond glycaemic control (90).

The World Health Organization (WHO) reports that the age-standardized adult diabetes prevalence was 9.8% in men and 9.2% in women in 2008, up from 8.3% and 7.5% in 1980. This represents an increase from 153 million in 1980 to 347 million in 2008(91).

In 2005, it was estimated that the prevalence of adult diabetes (Type 1 and Type 2) in Republic of Ireland was 4.7%, which equates to 141,063 adults(92). In 2010, data from Institute of Public Health of Ireland estimated that more than 185,000 (8.9%) adults age more than 45 years old have diabetes. By 2020, this number is predicted to rise by 30% to more than 175,000 (9.1%)(93).

1.2.2 T2DM and Cardiovascular Disease (CVD)

Today, macrovascular complications (CVD including stroke) are the most common cause of death in patients with diabetes(90).

Epidemiological and pathological data 1.5 decades ago firmly established diabetes as an independent risk factor for CVD in both male and female patients(94-96). When diabetes patients develop CVD, they have a poorer prognosis than non-diabetic subjects(97, 98) and are more likely to develop cardiomyopathy in the future(99, 100). There is a 3-fold increase in stroke mortality in patients with diabetes compared to non-diabetic subjects(101); with diabetes patients having an increased likelihood of severe carotid atherosclerosis compared to subjects without

diabetes(102, 103). More than half of participants commencing haemodialysis due to end stage renal disease have T2DM(104), and these patients' mortality is shown to be in excess of 20% per year(104) with CVD complications as the most common cause of death(105, 106).

The United Kingdom Prospective Diabetes Study (UKPDS) trial was a landmark trial in T2DM designed to investigate the relationship between glucose lowering intervention and risk of complications. Subjects were randomized to intensive glycaemic treatment vs standard treatment and followed prospectively. Standard treatment (n=1138) was diet alone with secondary randomization to active glucose lowering treatment if fasting plasma glucose was more than 15mmol/L. The intensive group was randomized to monotherapy treatment with insulin (n= 1156), sulphonylurea (n=1573) or metformin (in those with ideal bodyweight > 120%, n= 342). After a median follow-up of 10 years, there was a reduction of microvascular events of 25% in the intensive group (p=0.009). A similar trend was seen in macrovascular outcomes whereby intensive glycaemia control resulted in a 16% reduction in combined fatal and nonfatal myocardial infarction (MI) and sudden death. This however, failed to reach statistical significance with a p value of 0.052. There was also no suggestion of reduction in stroke.

In a 10-years follow up post trial, however, a significant reduction in MI and all cause mortality in intensively treated group were observed suggesting a legacy effect of early and aggressive glucose lowering treatment(107).

1.2.3 Relationship between treatment of T2DM and CVD mortality

The majority of patients with untreated T2DM will die from cardiovascular disease(108). To reduce this risk, both the American Diabetes Association (ADA) and the European Association for The Study of Diabetes (EASD) recommends individualized glycaemic control with a target HbA_{1c} of 53mmol/mol, targeted blood pressure and lipid control, in addition to managing other modifiable risk factors such as smoking and obesity(109, 110).

Over the last number of years, there have been new classes of antihyperglycaemic agents in the market. For the purpose of this thesis, I will only discuss the CVD risk with the use of metformin, sulphonylurea, thiazolidinediones, and dipeptidyl-peptidase-4 inhibitors.

1.2.4 General management in T2DM

Non-pharmacological treatment of T2DM involves extensive lifestyle modifications through a structured diabetes self-management education. These include practical and effective medical nutrition therapy, limitation of alcohol intake, moderate intensity aerobic physical activity (150 minutes per week), smoking cessation, psychosocial assessment and care, and appropriate immunization program(111).

Antihypertensive treatment should be considered in patients with office-based elevated blood pressure, with a target of 140/90mmHg in most patients; 130/80mmHg in younger patients with evidence of albuminuria and/or those with hypertension and one or more additional atherosclerotic CV risk factors, as long as no side effects such as hypotension. Other measures to reduce blood pressure such as weight loss, reducing sodium and increasing potassium intake, moderate alcohol intake, and increased physical activity should be emphasized(109).

Patients age less than 40 years old with CVD risk factors (LDL > 2.6mmol/l, hypertension, smoking, overweight and obesity) or overt CVD (previous cardiovascular events or acute coronary syndrome) need to be strongly considered for moderate or high dose statin therapy. Patients between the age of 40 to 75 years old with or without CVD risk factors or overt CVD are strongly recommended to receive either high intensity or moderate intensity statin therapy respectively(109).

Antiplatelet therapy such as aspirin is recommended as primary prevention in participants with increased cardiovascular risk (10-year risk > 10%). This would include most men above 50 or women above 60 years old with at least one additional major risk factor (family history of CVD, hypertension, smoking, dyslipidaemia, or albuminuria). Routine

screening of coronary artery disease in participants with T2DM is not recommended(109).

1.2.5 Metformin and CVD

When lifestyle modification alone has not achieved or maintained glycaemic target, a patient centered pharmacological treatment should be initiated. Metformin monotherapy has a proven evidence base for safety and efficacy. It is inexpensive and may reduce CV events(107). After 3 months, if there is no improvement in HbA_{1c}, a second agent may be added to metformin. Choices include sulphonylurea, thiazolidinedione, DPP4-inhibitors, SGLT2-inhibitors, GLP receptor agonist or insulin. Triple therapy combination with various antihyperglycaemic agents and further combination injectable therapy are considered in patients who fail to achieve glycaemic target(112).

The UKPDS Study published in 1998 provided the first robust outcome data about the efficacy and safety of the then-licensed T2DM treatment(108). Significant reduction of microvascular disease and a non-statistically significant reduction in myocardial infarction were seen in intensively treated participants with sulphonylurea or insulin compared to diet alone. In overweight participants (bodyweight greater than 120% ideal weight) randomly assigned to metformin, a 39% relative risk reduction of fatal and non-fatal myocardial infarction ($p=0.01$) and a 36% relative risk reduction in all cause mortality ($p=0.011$) were seen(113). In the 10-year follow up of UKPDS(107), these metformin-treated obese T2DM continued to show reduction in MI (33%) and death from any cause (33%). Despite a smaller cohort ($n=342$ on metformin, $n=411$ on diet alone), lack of lipid lowering drug and modern blood pressure and kidney preserving medications, these finding cemented the CV benefit of metformin and supported its adoption as a first line treatment in T2DM(108).

Most retrospective analyses of large databases(114-117), which suggested the CV benefit of metformin, used sulphonylurea as a comparator. Therefore, it is not possible to determine whether the

sulphonylurea increased, or metformin decreased, CV outcome in these analyses(118). In an example of a prospective study, Hong et al randomized 304 T2DM participants with a history of coronary artery disease to glipizide or metformin. After 5 years, metformin reduced the risk of the composite endpoint (CV death, any cause mortality, MI, non-fatal stroke, and arterial revascularization) by 46%(119). Two ongoing double-blinded RCTs (Metformin in CABG trial, MetCABG and GIPS-III Study) will determine if metformin can reduce infarct size and improve left ventricular function after ischaemia-reperfusion injury(118). A more definitive answer may be provided by the ongoing GLINT Study whereby 12 000 high-risk subjects with dysglycaemia but without overt diabetes are randomized to either placebo or metformin for 5 years(118).

There have been studies evaluating the effect of metformin on CVD proxies, most commonly carotid intima media thickness (C-IMT)(118). Earlier studies demonstrated small but significant decreases in C-IMT in participants treated with metformin compared to placebo(120-122). However, in the CAMERA study(123), metformin had no effect on C-IMT progression over 18 months in 173 non-diabetic participants although in contrast to earlier studies, all subjects in the CAMERA study were on statin which will minimize any effect of metformin(118). Previous studies have also demonstrated a potential benefit of metformin on endothelial function by reducing plasminogen activator inhibitor-1 (PAI-1) after 12-weeks of treatment compared to placebo(124).

In summary, the weight of the evidence supports a beneficial effect of metformin in reducing CV risk.

1.2.6 Sulphonylurea and CVD

Sulphonylureas (SUs) are insulin secretagogues used in T2DM since 1960s. SUs inhibit ATP-sensitive potassium channel in beta cells, which leads to depolarization, opening of calcium-gated channels, and calcium-dependent release of insulin by exocytosis(125).

These ATP-sensitive channels are also present in cardiac myocytes, which are normally closed. In chronic ischaemia, anaerobic metabolites

increase resistance in these channels to ATP, a cardioprotective mechanism called pre-conditioning. In the presence of SU, this preconditioning adaptation is blunted which is hypothesized to potentially contribute to more arrhythmias and larger infarct size with myocardial ischaemia(125).

The first study of SU and CV outcome was the University Group Diabetes Program (UGDP) trial in 1971. An older generation SU, tolbutamide was associated with increased CV death compared to placebo(126). This study was highly criticized as it was not designed nor powered to test the hypothesis of inferior CV safety for SU versus placebo(127) and the study was confounded by differences in baseline characteristics(125).

A different SU, Glibenclamide was studied in multiple randomized control trials (RCTs) and demonstrated increased cardiac pain compared to placebo, impaired cardiac pre-conditioning more than glimepiride and placebo (128, 129) and greater in-hospital mortality in participants with myocardial infarction compared to gliclazide(130).

In a recent review article, Rosenstock et al highlighted the discordance between the observational studies and RCTs of SU and its cardiovascular risk(127).

Some observational studies demonstrated increased all-cause and CV mortality with either SU monotherapy or when SU was combined with metformin, as compared to metformin monotherapy(115, 131). A meta-analysis of observational studies demonstrated that a combination of SU-metformin therapy versus either monotherapy was associated with increased risk of composite CV hospitalization or mortality (RR 1.43; 95% CI 1.1 – 1.85), but was not associated with increased risk in either outcome alone; which is difficult to explain(132). A nested sub-study (n=268) of UKPDS demonstrated an increased risk of diabetes-related death (96% increased risk, p=0.04) and all-cause mortality (60% increased risk, p=0.04) when metformin was added to SU in obese and non-obese participants compared to SU alone. These UKPDS investigators commented that this might represent extremes of chances

and epidemiological analysis of this possible association showed no increased risk in participants treated with SU/metformin combination therapy(113).

There are 15 published RCTs over 15-year period that were ≥ 72 weeks duration and included SU therapy vs active comparator/s or as part as treatment strategy(127). The UKPDS analyses involving first and second generation SUs, (UKPDS 33 and 80) and demonstrated that chlorpropamide, glyburide (glibenclamide) and glipizide were not associated with adverse CV events(107, 133). Similarly in the other 14 RCTs including the A Diabetes Outcome Progression Trial (ADOPT, n=4360), and ADVANCE study, a lack of CV adverse outcome was seen when SU was used in addition to other antihyperglycaemic agents(127). It must be noted that none of these studies had 'similar target levels' of glycaemic control in the treatment arm or were powered to demonstrate CV safety/benefit, and there were inconsistencies in how the CVD events were reported and adjudicated(127).

A dedicated RCT addressing the CV safety question of SUs such as the ongoing Cardiovascular Outcome Study of Linagliptin vs Glimepiride (CAROLINA) trial will hopefully be informative(127).

Gliclazide and Gliclazide MR (slow release) are the new-generation SUs in the market since early 2000. These medications are licensed to be used in Europe but not in the US(112). Compared to the other SUs, Gliclazide seems to be the most selective with respect to pancreatic receptor stimulation(134). A few observational studies showed cardiovascular benefits of gliclazide over other SUs(114, 135, 136) and it is the SU of choice in the most recent Netherlands T2DM management guidelines(134).

A recent systematic review and meta-analysis of RCTs comparing Gliclazide to other oral anti-diabetic drugs for at least 12 weeks (19 trials, n=3083 on gliclazide, n=3155 on other oral anti-diabetic) demonstrated lower hypoglycaemia episodes and similar effectiveness compared to other agents(134). However, the methodological quality of the RCTs was

poor and some were limited due to publication bias. None were designed for evaluating cardiovascular outcomes and this will require further attention in future RCT(134).

In summary, SUs are recommended as one of the next choice of second line agent after metformin. SUs have high efficacy, low cost and vast user experience by clinicians. As it stands, there are discrepancies in the evidences from retrospective and prospective studies for the CV safety/risk of SU use. A dedicated RCTs (CAROLINA) is currently ongoing to address the issue of CV risk/benefit in SU.

1.2.7 Thiazolidinediones and CVD

Thiazolidinediones (TZDs) are a group of drugs that exert their antihyperglycaemic effect by reducing insulin resistance in the peripheral tissue via activation of peroxisome proliferator-activated receptor gamma (PPAR- γ)(137). Troglitazone was removed from the market due to liver toxicity while Rosiglitazone and Pioglitazone carry a significant warning about their link to congestive heart failure (CCF)(112).

Earlier studies demonstrated a potential benefit of TZD on CV risk factors. TZD reduces peripheral tissue insulin resistance and this has been associated with a reduction in blood pressure(138). A meta-analysis of 27 studies demonstrated reduction of systolic blood pressure (SBP) by 4.7 mmHg and diastolic blood pressure (DBP) by 3.78 mmHg in participants on TZD(139). Rosiglitazone reduced triglycerides (TG) by 39% in one study(140) but in another study, increased TG by 15% while Pioglitazone reduces TG by 15%(141). Both Pioglitazone and Rosiglitazone have positive effect of increasing high-density lipoprotein (HDL) by about 8-15%(141). Both increase LDL by about 16-23% (less increase with Pioglitazone) and modify the LDL molecule into a larger, less atherogenic form of LDL(141). Both have been associated with a reduction in inflammatory markers and increased adiponectin levels, which may benefit CV profile as well(142).

Currently, the benefits of TZDs have been offset by concerns of oedema, CCF and long-term cardiovascular outcome. An incidence of oedema of

3-5% has been associated with the use of TZD monotherapy, 7.5% in combination of TZD and SU (vs 2.1% in SU alone) and 13-16% if using TZD in addition to insulin (vs 4-7% in those using insulin alone)(143). The incidence of CCF is much lower at less than 1% with Rosiglitazone monotherapy and 1.1% for Pioglitazone monotherapy and combination with insulin(143).

In 2007, a meta-analysis of 42 RCTs comparing the use of Rosiglitazone with placebo or active comparators of more than 24 weeks was published. The main purpose of this study was to assess the effect of this agent on cardiovascular outcome. Rosiglitazone was associated with significantly increased risk of myocardial infarction with OR 1.43 (95% CI 1.03 to 1.98, $p=0.03$) and a borderline significant risk of death from cardiovascular causes with OR 1.64 (95% CI 0.98 to 2.74; $p=0.06$)(144).

A further prospective study of 5-7 years duration in the RECORD Study (Rosiglitazone Evaluated for Cardiovascular Outcome in Oral Agent Combination Therapy for Type 2 Diabetes) however, did not suggest an increase in major adverse cardiovascular events (MACE) when Rosiglitazone was added to either metformin or SU(145). An independent re-adjudication of this data by the Duke Clinical Research Institute provided reassurance that Rosiglitazone was not associated with excess cardiovascular risk(146).

In the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROACTIVE), 5238 T2DM participants with macrovascular disease were randomly assigned to Pioglitazone or placebo for a mean of 34.5 months. There was no significant difference in the number of participants from each group who had at least one CV event ($p=0.095$). In the Pioglitazone group, there was a reduction in composite all-cause mortality, non-fatal MI and stroke by 16% (HR 0.84; 95% CI 0.72 to 0.98, $p=0.027$)(147). In a similar study, there were more cases of bladder neoplasm (14 vs 5), fewer cases of breast cancer (3 vs 11) in Pioglitazone group compared to placebo; and higher rate of bone fractures (5.1% vs 2.5%) in Pioglitazone treated female participants(148). In a more recent meta-analysis, the

incidence of bladder cancer in participants treated with Pioglitazone is 20.8 per 100 000 person years(149).

In 2010, the European Medicines Agency (EMA) suspended the use of Rosiglitazone. In US, it was subjected to enrollment into the Avandia-Rosiglitazone Medicines Access Program (REMS). The Food and Drug Administration (FDA) also issued an update to the Guidance for Industry in 2008 whereby prior to submission for new drug application, sponsors must compare the incidence of important cardiovascular events occurring with the investigational agent to the incidence of the same types of events occurring with the control group to show that the upper bound of the two-sided 95 percent confidence interval for the estimated risk ratio is less than 1.8. If the estimated increased risk is between 1.3 and 1.8, a post-marketing trial will be necessary to show that the upper bound of the two-sided 95 percent confidence interval for the estimated risk ratio is less than 1.3. If the premarketing application contains clinical data with estimated increased risk less than 1.3, a post-marketing cardiovascular trial generally may not be necessary(150).

In June 2013, the FDA re-evaluated the CV risk of Rosiglitazone based on the RECORD Trial (Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycaemia in Diabetes)(145). This multicentre, open labeled study randomly assigned 4447 poorly controlled T2DM patients to either Rosiglitazone or SU in addition to 1st line treatment. The hazard ratios in patients receiving Rosiglitazone were 0.95 for the composite end point for CV death, MI or stroke; 0.9 for CV death; 1.13 for MI; 0.79 for stroke; and 0.86 for all-cause death. None were statistically significant. Based on these non-inferiority findings, in May 2014, the FDA removed certain restrictions in prescribing Rosiglitazone.

In summary, Rosiglitazone and Pioglitazone are effective antihyperglycaemic drugs with additional benefits on blood pressure, lipid profile and inflammatory markers. Long-term studies and meta-analysis suggested increase risk of oedema, heart failure and possible risk of myocardial infarction and death. Previously, CV outcome was not a

requirement for drug approval but the Rosiglitazone experience has changed this. While this requirement is beneficial for patient safety, evaluation of CV outcome will potentially require higher cost, longer-term studies that will have an impact of the medication's value upon entering market.

1.2.8 Dipeptidyl-peptidase-4 inhibitors (DPP4i) and CVD

One of the newer classes of antihyperglycaemic medications are incretin-based medications. These include DPP4i and Glucagon like peptide-1 agonist (GLP1a). Glucagon like peptide 1 (GLP1) is secreted by intestinal cells and rapidly deactivated by DPP4 enzyme. Through specific receptors on pancreatic β -cells, GLP1 provides an additional stimulus to insulin secretion after oral glucose ingestion, that is not present with intravenous glucose infusion. It is well documented that patients with diabetes have impairment in the GLP1 response following ingestion of food(151). DPP4i increases endogenous GLP1 by inhibiting the function of the deactivating enzyme DPP4. Sitagliptin, Saxagliptin, and Linagliptin are among the DPP4i available in the market today. DPP4i reduce HbA_{1c} by approximately 0.5 to 1% and are weight neutral(3).

Earlier meta-analysis of RCTs in participants who were randomly assigned Saxagliptin in a broad drug development program demonstrated no increased risk of CV death, MI or stroke, with a relative risk reduction of 0.44 (95% CI 0.24-0.82)(152). Another meta-analysis of 41 RCTs of DPP4i use in T2DM for more than 12 weeks demonstrated a non-significant reduction in CV death and all cause of death with a relative risk of 0.76 (0.46-1.28) and 0.78 (0.4-1.51) respectively(153).

On the background of the new FDA regulation, the EXAMINE Study evaluated the CV outcome in 5380 T2DM participants with acute coronary syndrome (ACS) randomized to Alogliptin or placebo in addition to existing antihyperglycaemic and CV drug therapy. After a median follow up of 18 months (up to 40 months), the hazard ratio for composite of death from CV causes, nonfatal MI, or nonfatal stroke was 0.96 ($p < 0.001$ for non-inferiority)(154). Similarly, the SAVOR-TIMI study

evaluated the CV outcome in 16 492 T2DM participants who had a history of, or were at risk for CV events; randomly assigned to either Saxagliptin or placebo over a median of 2.1 years. There were no significant differences in the composite of CV death, MI, or ischaemic stroke (primary outcome) with HR of 1.0 with Saxagliptin (0.89-1.22, $p=0.99$ for superiority, $p<0.001$ for non-inferiority). Unexpectedly, more participants in the Saxagliptin group were hospitalized for heart failure with a HR of 1.27 (1.07-1.51, $p=0.007$)(155). The increase in risk was highest among participants with elevated level of natriuretic peptide, previous heart failure, or chronic kidney disease. From a safety viewpoint, more patients in the Saxagliptin group (15.3%) had hypoglycaemic events compared to the placebo group (13.4%, $p<0.001$). There were similar risk of cancers, pancreatitis, haematological disorders, infections, skin reactions, bone fractures and abnormal liver function.

Both of these studies overturned the previous meta-analyses showing CV benefit of DPP4i, and instead demonstrated a non-inferiority of DPP4i with regards to CV safety, compared with placebo, when added to usual diabetes care(108).

In summary, DPP4i improve HbA_{1c}, are well tolerated, and with a weight neutral effect, a welcome addition as a new class of antihyperglycaemic agent. The long-term effects of DPP4i on CV risk or benefit are still unclear. An alternative method of identifying this risk or benefit is needed in order to prevent similar experience seen with Rosiglitazone. There are at least 4 other ongoing longer term RCTs evaluating CV outcome in DPP4i (TECOS, CANVAS, BI10773, and CAROLINA) and hopefully these will shed further light on potential risk and benefit of this group of antihyperglycaemic drugs with longer term use.

1.2.9 Endothelial Dysfunction in T2DM

Insulin resistance and hyperinsulinaemia, adipose tissue, oxidized LDL, reactive oxygen species and advanced glycation end product (AGE) are factors affecting endothelial function in T2DM.

Insulin resistance is common in T2DM and it can be present for many years before any abnormality of plasma glucose(156). It is defined as decreased ability of insulin to promote glucose uptake in skeletal muscle and adipose tissue and to suppress hepatic glucose output. Animal and human studies of insulin resistance have demonstrated the role of insulin resistance in endothelial dysfunction (157) which is a precursor to atherosclerosis(68).

Insulin resistance may be linked to endothelial dysfunction by a numbers of mechanisms (Figure 1-3). Increased adiposity results in increased production of free fatty acids (FFA) due to increased lipolysis. FFA decrease glucose oxidation and inhibit mobilization of GLUT4 (major glucose transporter), which further inhibits insulin-mediated glucose uptake(158).

Adipose tissue also produces TNF- α and resistin which further mediates insulin resistance(159). Over time, these lead to further development of metabolic syndrome characteristics, such as development of hypertension, hyperuricaemia, low HDL cholesterol, production of plasma Plasminogen Activator Inhibitor 1 (PAI-1) mediated thrombotic tendency and elevated of oxidation-prone, small dense LDL cholesterol(160). These components of metabolic syndrome increase vascular production of reactive oxygen species which further worsen endothelial dysfunction(160). Hyperinsulinaemia also enhances neutrophil transendothelial migration, which leads to increased production of platelet endothelial cell adhesion molecule-1 (PECAM-1) but not ICAM-1, P-selectin or E-selectin(161).

Oxidized LDL cholesterol disrupts the cholesterol-rich invaginations on endothelial cells and vascular smooth muscle cells called caveolae(157). Endothelial nitric-oxide synthase (eNOS) interacts with caveolae and

decreases vasoconstrictive effect of angiotensin II and endothelin. The disruption of caveolae is thought to be the mechanism that reduces eNOS availability and contributes to endothelial dysfunction. HDL cholesterol however prevents the oxidized LDL-mediated decrease cholesterol in caveolae and donates cholesterol to the caveolae complex, which further improves the endothelial responsiveness to Acetylcholine (ACh)(157).

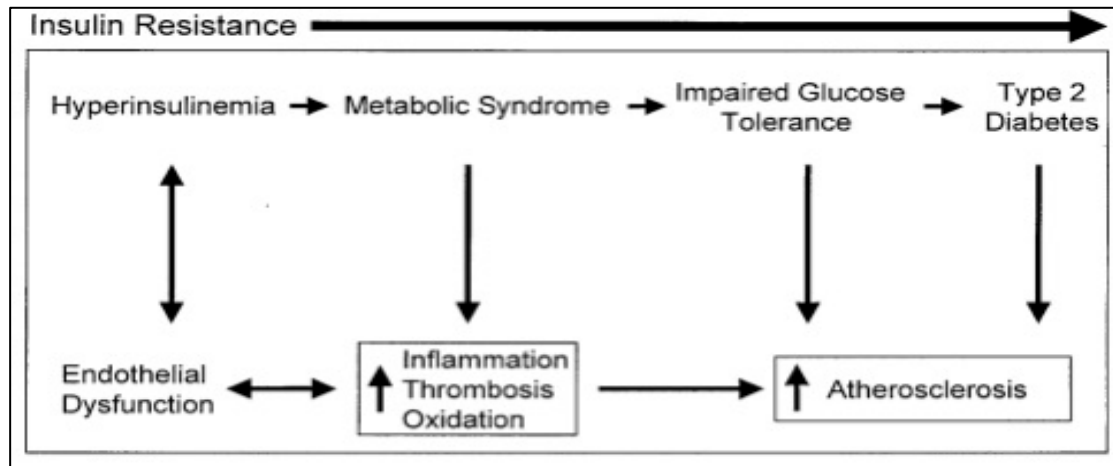


Figure 1-3: Progression of insulin resistance to T2DM parallels the progression of endothelial dysfunction to atherosclerosis. Adapted from Hsueh et al Am J Med.2004;117:109-117.

Advanced glycation end-product (AGE) consists of protein or lipids that became glycated after exposure to a hyperglycaemic state as in diabetes mellitus. Mechanisms accompanying AGE mediated inflammation are likely multifactorial. Besides promoting oxidized-LDL, through the diacylglycerol (DAG)-protein kinase C (PKC) pathway, AGE lead to superoxide particles that inactivate NO production which leads to accelerated atherosclerosis(162).

These multiple insults on endothelial cells results in lost of cell integrity, cell senescence and further apoptosis(163, 164). Loss of cell integrity produces a paradoxical constriction instead of vasodilatation when endothelial cells exposed to increasing amount of ACh. Additionally,

through a complex interaction involving mechanical stretch of β 1-integrin signaling pathway, an apoptotic process is induced(68).

Apoptosis leads to detachment of endothelial cells, which are released into the bloodstream and can be recognized and measured as circulating endothelial cells irrespective of glucose level(165). Besides endothelial cells, endothelial microparticles released from the intact cells following apoptosis act as a powerful pro-coagulant and have been shown to be predictive of coronary artery lesion, and more significant independent risk factors than duration of diabetes, lipid levels and presence of hypertension(166). Arterial denudation is the end result of endothelial cells apoptosis. This process triggers smooth muscle proliferation, migration and matrix secretion which are pro-atherosclerotic(68).

The capabilities of EPC to home in on ischaemic sites, differentiate into mature endothelial cells, and form a cellular patch at sites of endothelial injury contributes directly to homeostasis and repair of endothelial layer(68). This reparatory process is fundamental in re-establishing vessel integrity. With almost all risk factors for atherosclerosis having been associated with decrease and/or dysfunction of EPC, it plays a major role in cardiovascular biology and provides a mirror image of cardiovascular health(167).

In summary, the pathogenesis of endothelial dysfunction in T2DM is multifactorial. The insults to endothelial cells began with the development of insulin resistance before diabetes is diagnosed. The end results are endothelial cells apoptosis and arterial denudation with atherosclerosis as a consequence. There is increasing evidence that EPC provides an essential role in endothelial cell reparatory process and improvement of EPC numbers and/or function may help to reduce the risk of atherosclerosis.

1.2.10 EPC in T2DM

Multiple studies have demonstrated a reduction in the number of EPC by up to 40% and 44% in T2DM and T1DM patients compared to controls. Diabetes also affects the angiogenic function of EPC in vitro especially in the formation and participation of endothelial tubule formation (42, 168, 169).

Some researches suggest that level of EPC may fluctuate as the patient progresses through stages of diabetes. In a study involving 425 subjects with various degree of glucose metabolism status (normal glycaemia to diabetes), and with different diabetes duration, demonstrated a significant nadir of CD34⁺ EPC at the time of diagnosis of T2DM, partial recovery in the subsequent years (up to 19 years post diagnosis), followed by exhaustion of bone marrow reserve in the longer term (> 20 years post diagnosis) (170). Using multiple linear regression models, the reduction in EPC is partly attributed to aging, concomitant risk factors or CVD and not entirely to their carbohydrate metabolism status or diabetes duration. In addition to weak marrow mobilization, decreased proliferation and shortened survival, diabetic EPCs demonstrated impairment in proliferation, adhesion, and migration at the damaged endothelial cells(171).

The mechanisms underlying reduction of EPC in diabetes patients seems to include reduced bone marrow mobilization, reduced proliferation and shortened survival of EPC(172).

Central to reduced marrow mobilization of EPC in diabetes is a defect in marrow stimulation following ischaemia. Tissue ischaemia is considered the strongest stimulus for EPC release from bone marrow(172). The hypoxia sensing system HIF-1 (hypoxia-inducible factor-1) consists of heterodimeric transcription factor composed of α (HIF-1 α) and β (HIF-1 β) subunit. In cellular hypoxia, the normal degradation process of HIF-1 α is attenuated allowing dimerization with HIF-1 β resulting in active HIF-1. This will bind to enhancer DNA regions and promotes transcription of oxygen sensible genes that encode, among others VEGF, SDF1 α and

erythropoietin (EPO)(173). These growth factors stimulate EPCs into the circulation by transendothelial migration, and further recruitment is mediated by hypoxic gradient via HIF-1 induced SDF1 expression(67). In a streptozotocin-diabetes rat model with hind-limb ischaemia, stimulation of EPC mobilization following ischaemia was impaired. Additionally, these diabetes rats had altered release of SDF-1, VEGF and inability to upregulate muscle HIF-1 α compared to control rats(174). This HIF-1 α – SDF-1 – VEGF hypoxia responsive system has been shown to mediate adhesion, migration and homing of circulating progenitor cells to ischaemic tissue(67). The weak angiogenic response through this system in the setting of tissue ischaemia results in the inability to mobilise the progenitor cells necessary for new vessel formation(172).

Reactive oxygen species (ROS) have been implicated in the deregulation of HIF-1 α . Further on, inhibition of these ROS by N-Acetylcysteine (NAC) in diabetic mice with ischaemia restored the endothelial growth factors level to non-diabetic level and up-regulates the number of EPC(175).

Insulin treatment in T2DM may increase the number of EPC as demonstrated in an in vivo study involving poorly controlled diabetes patients (n=23). In this study, EPC numbers improved by 65% following insulin treatment, and this did not correlate with the improvement in HbA_{1c} and fasting glucose. Subjects were further screened for SDF1 genotypes and following multivariate analysis, SDF1-3'A/G genotype was an independent predictor of EPC mobilization following insulin therapy(176). Nevertheless, it can be postulated that improvement in metabolic control may induce more efficient stimulation of EPC-mediated neovascularization(68).

Another mechanism underlying weak marrow stimulation is alteration of the PI3K/Akt pathway in patients with diabetes. Diabetes leads to hyperglycaemia and this generates the non-enzymatic glycation of protein and lipids (AGE), which accumulate in the vessel wall. AGE binds to its transmembrane receptor (RAGE) producing a cascade of cellular events involving protein kinase C (PKC) and mitogen-activated protein kinase

(MAP), which leads to cellular dysfunction(177). Diacylglycerol (DAG) and PKC are essential intracellular signaling molecules responsible for vascular permeability, vasodilator release, endothelial activation and growth factor signaling. Pathological activation of PKC by DAG occurs in hyperglycaemia resulting in increased permeability of vessel wall, altered NO regulation, increase leukocyte adhesion, and enhanced expression of growth factors (VEGF, TGF- β , ET-1)(177). These intracellular activations of PKC and MAP are part of a bigger system of intracellular signaling mediated by P13K/Akt pathway and this pathway have been suggested to mediate EPC endothelial migration(41).

Hyperglycaemia also alters leukocyte and endothelial function through the production of reactive oxygen species (ROS)(178) and may have a role in the apoptosis of EPC. In an in vitro study, EPC from healthy donors were exposed to hyperglycaemia and normoglycaemia state. Hyperglycaemia state resulted in increase in apoptosis and reduction in EPC proliferation compared to normoglycaemia state. This oxidative stress also plays a significant role in damaging the interaction between vascular wall and EPCs while induction of the antioxidant, hemeoxygenase-1 (HO-1) gene partially reverses this process(179). Elevated C-reactive protein (CRP) and systemic hypoxia have been shown to downregulate EPC by enhancing apoptosis(180, 181) while gene expression of NADPH oxidase, which is a major source of ROS, is increased in EPC precursor monocytes from T2DM patients depending on glycaemic control(182).

Hyperglycaemia state in diabetes has been shown to cause functional impairment as reflected by reduction in NO production and metalloproteinase (MMP)-9 activity, as well as impairment in migration and integrative capacities(183). The evidence in vivo is not conclusive and needs further investigations(68). A study involving subjects with varying degree of diabetes (IFG, IGT and T2DM) including non-diabetes subjects demonstrated an inverse relationship between EPC apoptosis and peripheral EPC level with no difference in the apoptotic rate between diabetes and non-diabetes subjects(170).

Metabolic syndrome (MetS) and insulin resistance in diabetes reduces EPC numbers especially CD34⁺ progenitor cells. In a cross-sectional study of 214 subjects with variable degree of CV risk, all components of MetS has a negative impact on the number of CD34⁺ cells and clusters of these factors act synergistically to reduce the numbers of these cells. In a subgroup analysis of 125 subjects, the measurement of insulin resistance homeostatic model arrangement (HOMA) was negatively correlated with CD34⁺ cell counts indicating potential molecular mechanisms in patients at risk of diabetes(38). Furthermore, insulin resistance state in metabolic syndrome is characterized by defective activation of P13K/Akt pathway and decrease endothelial NO activity which are essential for EPC mobilization and function(172).

In summary, diabetes affects the release, mobilization, function and survival of EPC through various pathways. Hyperglycaemia, hyperinsulinaemia, oxidative stress and metabolic syndrome are some of the known pathological changes common in diabetes, and these factors are having a negative impact on EPC.

1.2.11 Effect of Anti-diabetic Treatment on EPC

Anti-diabetes medications have been shown to have a positive effect on EPCs.

1.2.11.1 Sitagliptin and EPC

Fadini et al evaluated the effect of Sitagliptin for 4 weeks compared to no treatment (control) on the level of EPC in T2DM patients already treated with metformin and/or secretagogues(184). EPC were counted using flow-cytometry and identified with their surface marker CD34⁺KDR⁺ cells. Total CD34⁺ count, expression of CXCR4, SDF1a, VEGF, MCP-1, NO and plasma DPP4 were measured.

In this study, there was two-fold increased in circulating EPC following Sitagliptin therapy compared with control. Also there was an increased plasma concentration of SDF1a by 50% and a reduction in plasma Monocyte Chemoattractant Protein-1 (MCP-1) by 25% in Sitagliptin group compared to no treatment. There were no significant changes seen in NO

level or the level of CD34⁺ cells counts in both groups. SDF1a receptor CXCR4 were only expressed on 17% of CD34⁺ cells compared to 63% of CD34⁺KDR⁺ cells which explains the differential effect of Sitagliptin on either cells. It is postulated that this beneficial effect on EPC was mediated by augmenting the level of SDF-1 α , which has been shown to be essential in EPC mobilization from bone marrow, migration, proliferation, and survival (65-67, 184).

1.2.11.2 Saxagliptin and Proangiogenic Cells (PACs)

Poncina et al evaluated the effects of Saxagliptin on the pro-angiogenic cells (PAC) including cells considered to be EPC, in a cross-sectional study involving healthy volunteers and patients with T2DM(185). PACs isolated from peripheral blood were incubated after day 4 with an equivalent therapeutic 5mg dose of Saxagliptin and/or SDF1 α in-vitro. Proliferation, clonogenesis, adhesion, migration and tube formation were analyzed. Gene expressions analyses on PACs were performed in both groups at baseline. The effect of Saxagliptin and/or SDF1 α on PACs gene expression was performed on a subgroup of PACs CD14⁺ and CD14⁻ from healthy volunteers (n=20) only. Finally, a separate in-vivo study assessing the pro-angiogenic effects of Saxagliptin was performed using PACs isolated from T2DM patients who had been treated with Saxagliptin (>4 months at enrolment, n=5) and this was compared to T2DM patients not treated with Saxagliptin.

In this study(185), Saxagliptin and/or SDF1- α did not restore PACs colony formation in patients with T2DM but tended to increase the colony numbers in healthy controls. Similarly, co-treatment with SDF1- α promoted haematopoietic clonogenesis in healthy controls but not in PACs from T2DM patients. The different findings in these two groups may be related to lower baseline gene expression of CDKN1A and BCL2 genes in PACs from T2DM subjects. Saxagliptin was also shown to have no effect on the percentage of cells staining with acLDL⁺Lectin⁺(185)

Poncina et al used the Human Umbilical Vein Endothelial Cell (HUVEC) to assess adhesion capacity. Saxagliptin in the presence of SDF1- α

increased PACs adhesion in T2DM patients while it had no effect on PACs from healthy volunteers. There was no difference in PACs cells adhesion capacity when treated with Saxagliptin or SDF1- α alone in patients with T2DM(185). This indicates the important role of available SDF1- α in order to modulate the adhesion function of PACs.

Poncina et al also demonstrated that PACs from diabetic subjects had increased expression of DPP4 gene with lower expression of adhesion genes such as Vascular Adhesion Molecule 1 (VCAM1), Intercellular Adhesion Molecule 1 (ICAM1) and Integrin Subunit Beta 2 (ITGB2) compared to healthy control PACs. This may translate into an increase in DPP4 activity in-vivo in patients with T2DM independent of improvement in glycaemic control as demonstrated by Fadini et al(186). With SDF1- α acting as a physiological substrate for DPP4, an increase in DPP4 activity in PACs from T2DM subjects inactivates SDF1a (184).

1.2.11.3 Metformin, Gliclazide and EPC

Chen et al randomized 47 new T2DM patients to either Metformin monotherapy or Metformin and Gliclazide MR dual therapy for 16 weeks. Early dual therapy significantly improved circulating EPC numbers measured using flow cytometry and cultured EPC stained positive for acLDL and Lectin. Dual therapy also improved EPC migratory capacity and plasma markers of oxidative stress. There was no difference in the numbers of EPC colony forming unit in both groups(187). Metformin exerts its effect through the activation of AMP-activated protein kinase (AMPK) pathway(188, 189). Nitric oxide (NO) is essential in EPC mobilization and the AMPK pathway has been linked to the activation of endothelial cells stimulated endothelial nitric oxide synthesis (eNOS) and NO production(190). Gliclazide is a second-generation sulfonylurea with azabicyclo-octyl ring, described as having antioxidant property and has been shown to significantly improve EPC numbers and endothelial function in newly diagnosed T2DM patients compared to baseline(191).

1.2.11.4 Pioglitazone and EPC

Esposito et al examined the effects of Pioglitazone or Metformin on EPCs and markers of endothelial injury Endothelial Microparticles (EMPs), in 110 newly diagnosed T2DM. There were increased in the numbers of EPC with a reduction EMP in the Pioglitazone group compared to Metformin group after 24 weeks of therapy(192). It is postulated that by suppressing inflammation and improving the level of adiponectin, Pioglitazone may have a positive effect on EPC(193-195).

1.2.12 Summary

The prevalence of T2DM is increasing in Ireland and around the world. Multifactorial alterations in diabetes contribute to endothelial dysfunction that leads to atherosclerosis and macrovascular complications. Aggressive glycaemic, lifestyle modification, antihypertensive, statin and antiplatelet are essential. In general, anti-diabetic medications have positive effects on EPC. Metformin reduces CV events while the Sulphonylureas carry a neutral CV risk. DPP4i are one of the newer classes of antidiabetic medication with no CV outcome data at the initiation of this research. While the use of Pioglitazone appeared to be safe, the withdrawal of Rosiglitazone suggest a need for identification of CV risk or benefit prior entering the market, either with a long term study or by utilizing an appropriate CV marker. As EPC numbers predicts the future risk of CVD, estimation of EPC number may have a role in fulfilling this need in patients with T2DM.

1.3 Arterial Stiffness

As well as acting as a conduit for blood flow from the heart, arteries, and in particular the aorta, act as a 'dampening mechanism', to reduce the effect of myocardial contraction on changes in blood pressure and therefore resulting in a more steady flow of blood into peripheral tissue(196).

Arterial stiffness is an age-related process, that is a shared consequence of many diseases such as diabetes, hypertension, metabolic syndrome and chronic kidney disease among others(197). As aorta stiffens, it is less able to accommodate volumes of blood from the left ventricle, and this dampening mechanism is attenuated resulting in left ventricular hypertrophy and fibrosis, fluctuation of blood pressure, reduction in vessel wall shear stress and production of NO, and further promotes development of atheromatous plaques(196). Arterial stiffness has been shown to be a strong independent predictor of future CV events and all cause mortality in general population(6).

1.3.1 Assessment of Arterial Stiffness

Measurement of pulse wave velocity (PWV) especially the carotid-femoral PWV (also known as aortic PWV) is considered the gold standard in laboratory assessment of arterial stiffness(196, 198). With ventricular contraction, a pressure pulse is generated and this is propagated along the arterial tree. The speed of the pulse is determined by the geometric and elastic properties of the arterial wall and majority of this 'buffering' occurs in the aorta. A stiff artery results in an increase in pulse speed compared to an elastic artery. If the time interval between the foot of the pulse wave is measured transcutaneously at two different sites and the distance between the two sites is known, PWV can be calculated as: $\text{velocity (m/s)} = \text{distance (m)} / \text{time (s)}$ (196).

PWV is non-invasive, easy to perform, reproducible, and has been shown to predict CV events in large epidemiological and clinical studies(198-200). Two probes or automated cuffs are placed around the carotid at the neck and femoral artery at the groin. Using software from Complior®,

SphygmoCor® or Vicorder®, waveforms from common carotid and femoral artery will be generated (Figure 1-4). Time delay (or transit time) between the feet of both waveforms is measured and by measuring the distance between the two waveforms, a PWV is generated(198).

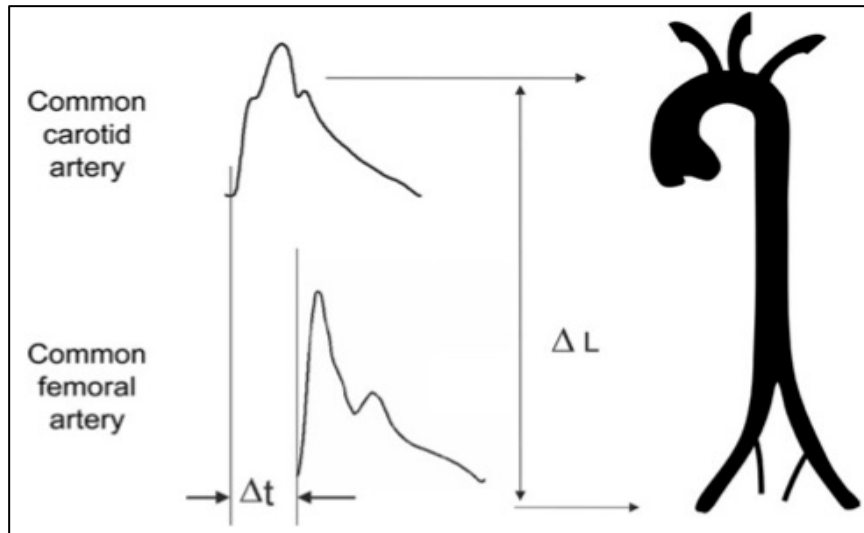


Figure 1-4 Measurement of carotid femoral Pulse Wave Velocity (PWV). Δt = transit time or time delay, ΔL = length or distance. Adapted from Laurent et al European Heart Journal (2006) 27, 2588–2605. DOI: 10.1093/eurheartj/ehl254

Recently, recommendations have been published in order to optimize PWV measurements(196, 201). It should be performed in a quiet room with stable temperature. Measurements should be performed in a supine position after 10 minutes of rest, preferably using the right common carotid and right common femoral artery. If repeated measurement is needed, it should be done at the same time of the day to avoid diurnal variation. Subjects should refrain from meals, smoking or caffeine within 3 hours of measurement, and from speaking or sleeping during measurements.

Data should be the mean of recorded pulse waves during at least one respiratory cycle (about 5-6s). Carotid femoral distance should be measured in a straight line. Measurement should be taken twice and the mean of it is calculated. If the differences between the two measurements

are more than 0.5m/s, perform a third measurement and the median value should be used(196, 201).

Despite being considered as a gold standard measurement of arterial stiffness, measurement of PWV in clinical practice is hampered by the lack of method standardization and reference value(202). In 2007, the European Society of Hypertension (ESH) and European Society of Cardiology recommended 12m/s as a threshold that signified alteration of arterial function in middle-aged hypertensive patients(203). However, this was based on epidemiological studies and could not take into account multiple factors influencing PWV(202). The most important factors consistently showing independent association with PWV are age and blood pressure(204, 205). A more recent consensus statement however reduced the estimate threshold to 10m/s by taking into account the 20% shorter true anatomical distance travelled by the pressure wave(201).

In 2010, The Arterial Stiffness Collaboration Group published the reference value for carotid femoral PWV for normal European population with no CV risk factors and proposed a reference value in population with various risk factors based on age and blood pressure categories(202). This consensus included 13 centres in 8 European countries with a total of 11 092 subjects (Table 1-1). Following that, Van Bortel et al published a new guideline that categorizes levels of aortic PVW associated with increased CV risk(201).

Table 1-1 Distribution of PWV values (m/s) in the reference value population (11 092 subjects) according to age and blood pressure category. SD (standard deviation), 10th percentile), 90th percentile), HT (hypertension). (Adapted from Eur Heart J 2010, Vol 31, Issue 19, 2338-50, DOI 10.1093/eurheartj/ehq165)

Age category (years)	Blood pressure category				
	Optimal	Normal	High normal	Grade I HT	Grade II/III HT
PWV as mean (\pm 2 SD)					
<30	6.1 (4.6–7.5)	6.6 (4.9–8.2)	6.8 (5.1–8.5)	7.4 (4.6–10.1)	7.7 (4.4–11.0)
30–39	6.6 (4.4–8.9)	6.8 (4.2–9.4)	7.1 (4.5–9.7)	7.3 (4.0–10.7)	8.2 (3.3–13.0)
40–49	7.0 (4.5–9.6)	7.5 (5.1–10.0)	7.9 (5.2–10.7)	8.6 (5.1–12.0)	9.8 (3.8–15.7)
50–59	7.6 (4.8–10.5)	8.4 (5.1–11.7)	8.8 (4.8–12.8)	9.6 (4.9–14.3)	10.5 (4.1–16.8)
60–69	9.1 (5.2–12.9)	9.7 (5.7–13.6)	10.3 (5.5–15.1)	11.1 (6.1–16.2)	12.2 (5.7–18.6)
\geq 70	10.4 (5.2–15.6)	11.7 (6.0–17.5)	11.8 (5.7–17.9)	12.9 (6.9–18.9)	14.0 (7.4–20.6)
PWV as median (10–90 pc)					
<30	6.0 (5.2–7.0)	6.4 (5.7–7.5)	6.7 (5.8–7.9)	7.2 (5.7–9.3)	7.6 (5.9–9.9)
30–39	6.5 (5.4–7.9)	6.7 (5.3–8.2)	7.0 (5.5–8.8)	7.2 (5.5–9.3)	7.6 (5.8–11.2)
40–49	6.8 (5.8–8.5)	7.4 (6.2–9.0)	7.7 (6.5–9.5)	8.1 (6.8–10.8)	9.2 (7.1–13.2)
50–59	7.5 (6.2–9.2)	8.1 (6.7–10.4)	8.4 (7.0–11.3)	9.2 (7.2–12.5)	9.7 (7.4–14.9)
60–69	8.7 (7.0–11.4)	9.3 (7.6–12.2)	9.8 (7.9–13.2)	10.7 (8.4–14.1)	12.0 (8.5–16.5)
\geq 70	10.1 (7.6–13.8)	11.1 (8.6–15.5)	11.2 (8.6–15.8)	12.7 (9.3–16.7)	13.5 (10.3–18.2)

1.3.2 Arterial Stiffness, PWV and T2DM: Clinical Studies

In 2014, Prenner et al published the first systematic review of studies evaluating arterial stiffness in patients with diabetes mellitus(197).

Besides carotid femoral PWV (cfPWV), other methods used to assess arterial stiffness were carotid-radial PWV (crPWV), carotid-ankle PWV (caPWV), pulse pressure (pp), and augmentation index (Aix).

Several studies have demonstrated increased in PWV in patients with diabetes when compared to non-diabetic controls(206-208). Diabetes patients have increased PWV measured at 4 segments of main vessels (heart-carotid, heart femoral, heart-brachial, and femoral-ankle) when compared to control, with greater effects of diabetes and age on larger vessels (heart-carotid and heart femoral)(206). Additionally, they also have an accelerated progression of arterial stiffness compared to normal subjects as highlighted in a study involving 169 subjects (n=57 diabetics, n=112 non-diabetics), whereby on average, diabetes subjects had PWVs (cfPWV, crPWV) readings similar to non-diabetics who were 15 years older(207). Evidence for this accelerated process is further provided by a

prospective studies in metabolic syndrome subjects, a known predisposing factor for T2DM development; whereby an increased in PWV seen with increasing number of metabolic syndrome components, over 6 and 9 years follow up(209, 210).

Studies examining the impact of arterial stiffness in diabetes have demonstrated links to end-organ damages including nephropathy, retinopathy, autonomic dysfunction/neuropathy, cognitive dysfunction and stroke(197).

Albuminuria precedes diabetic nephropathy, carries a poor prognostic risk and is an independent predictor of all-cause and cardiovascular mortality in patients with diabetes(211, 212). Smith et al evaluated the relationship between cfPWV and varying degree of albuminuria in 134 T2DM patients. Patients with raised albumin creatinine ratio (ACR) had stiffer arteries as demonstrated by higher cfPWV. There was a negative univariate association between cfPWV and GFR, suggestive of early changes in the viscoelastic property of large arteries well before the onset of overt diabetic nephropathy(213). Additionally, the independent association between aortic PWV with the level of albuminuria was demonstrated by Ishimura et al in a study involving 167 T2DM patients(214). Kimoto et al also demonstrated this independent association in larger study involving 434 T2DM patients and 192 healthy controls whereby stepwise progression of large artery stiffness was seen as GFR worsened after adjustment for confounders(206). The worsening GFR will lead to end-stage renal disease (ESRD) and aortic stiffness has been shown to predict mortality in subjects who progress to ESRD(200).

Another predictor of CV mortality in T2DM is the presence of diabetic retinopathy(215). In a cross-sectional study involving 494 T2DM patients, heart-femoral PWV (hfPWV) was significantly higher in patients with retinopathy compared to patients without retinopathy. There was also an independent association between quartile of hfPWV with retinopathy after adjustment for confounders(216).

Autonomic neuropathy is also associated with arterial stiffness and is a predictor of CV mortality(217). In a cross sectional study involving 45 T2DM patients and control subjects, PWV and autonomic function tests were performed. There was increase in central PWV in diabetes patients compared to controls with an independent positive correlation between PVW and autonomic neuropathy score(218). Larger studies involving 676 T1DM patients compared to controls demonstrated an increase in PWV with degree of cardiac autonomic neuropathy measured using heart-rate-variability (HRV) and the relationship persisted after adjustment for confounders(219).

Brain white matter lesions (WML) are associated with an increased risk of stroke(220). Laugesen et al investigated the association between cfPWV and the degree of WML in 89 T2DM patients compared with 89 controls. In this study, cfPWV was higher in diabetes patients despite having excellent glycaemic control and lower blood pressure and cholesterol profile compared to controls. The odds of having higher degree of WML in the brain was increased by 30% per 1m/s increased in cfPWV independent of potential confounders suggestive a potential role of PWV evaluation in risk stratification for stroke(221).

1.3.3 EPC and PWV in Diabetes Patients

There have been some limited studies evaluating the relationship between EPC and PWV in patients with diabetes.

Palombo et al used multiple measures of arterial stiffness (including cfPWV) and measured circulating EPC in 16 uncomplicated young T1DM patients and 26 controls. EPC numbers and cfPWV were lower in T1DM patients compared to controls. However, there was no significant relationship between circulating EPC numbers and cfPWV in the univariate analysis(222).

Yue at al evaluated EPC numbers and brachial-ankle PWV (baPWV) in 234 T2DM patients and 121 controls. Poorly controlled diabetes patients had lower EPC counts and higher baPWV compared to patients with good glycaemic control. Circulating EPCs were negatively correlated with

baPWV while multivariate analysis demonstrated HbA1c and EPC count as independent predictors of baPWV(223).

Protopsaltis et al evaluated cfPWV and EPC in 53 subjects with pre-diabetes. In this study, there was no correlation between cfPWV and EPC numbers(224).

In summary, arterial stiffness is an important predictor of CV disease especially in patients with diabetes. Measurement of carotid-femoral PWV is considered the gold standard in the evaluation of arterial stiffness and the latest evidence on PWV reference range and its relationship with CVD will strengthen its use in risk factor evaluation. Several clinical studies have proven the link between diabetes complications, arterial stiffness and CVD. However, there are limited data on the association between PWV and EPC in patients with diabetes.

1.3.4 Summary

Arterial stiffness is common in diabetes and is a strong independent predictor of future CV events and mortality. PWV is considered the gold standard in the evaluation of arterial stiffness. It is not until recently that we have a minimum PWV threshold. Arterial stiffness is more prevalent in diabetics compared to non-diabetics. It predicts the development of microalbuminuria, retinopathy, neuropathy and stroke.

1.4 Taurine

1.4.1 Discovery and significance of Taurine

Taurine, 2-aminoethanesulfonate is a free amino acid, present ubiquitously in millimolar concentrations in all mammalian tissues(9), with highest concentration in the heart, retina, brain, skeletal muscle, white blood cells and platelet(225).

The physiological role of taurine became more evident when cats and primates fed with taurine-free diet develop retinal degeneration(226, 227). Similarly, taurine supplementation in rats prevents conjunctiva, corneal and retinal changes typical of Xerophthalmia(228).

Humans have very low capacity to synthesize taurine and subsequently rely on dietary taurine, mostly from seafood and meat. A WHO worldwide epidemiological study demonstrated an inverse correlation between urinary taurine excretion, surrogate marker for dietary taurine intake and mortality rate caused by ischaemic heart disease(8).

Taurine has an essential role in antioxidation, modulation of ion movement(229), osmoregulation(230), modulation of neurotransmitters and conjugation of bile acids(231). In various animal models, the antioxidant property of taurine is essential to protect from diabetes and complications(9).

Taurine acts poorly with superoxide, peroxide and hydroxyl radicals. In the presence of activated neutrophils, Taurine reacts at 1:1 ratio with the hydrogen peroxide-myeloperoxidase-chloride (HClO) system forming taurine chloramine, acting as a scavenger for acid(232) in many cell types including myocardium (Figure 1-5).

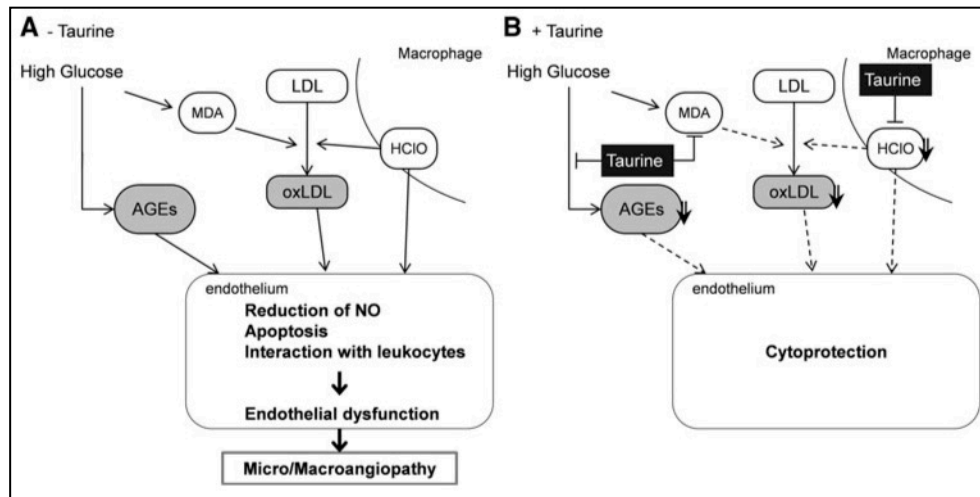


Figure 1-5: The beneficial action of Taurine against hyperglycaemia induced endothelial dysfunction. Taurine can inhibit AGE production, oxidized LDL (oxLDL), production through scavenging melondialdehyde (MDA) and hypochlorous acid (HClO), HClO-dependent NO reduction, and leukocyte-endothelium interaction. (Adapted from T.Ito et al. Amino Acids (2012) 42:1529-1539)

Oxidation of LDL is a myeloperoxidases (MPO) driven process, resulting in vasoconstriction by consumption of nitric oxide (NO)(233). It has been shown to be an important inflammatory marker in hyperglycaemia-mediated vascular inflammation(234), coronary artery disease and may have implications for atherosclerosis(235). By decreasing the carbonyl group production and reacting to aldehyde(9), the scavenger role of taurine reduces the oxidative damage by reducing the modification of LDL and increasing the bioavailability of NO(236).

This mechanism together with improvement in vascular tone may contribute to the role of taurine in hypertension management(10, 11, 237). In a recent double blind, placebo controlled study involving 120 untreated pre-hypertensive and 58 age-matched controls, 12-weeks taurine supplementation, significantly decreased the clinic and 24 hour ambulatory blood pressure measurement, and also improved endothelium dependent and independent vasodilation(11).

Similar mechanisms may also play a role in various in-vitro studies involving rats and human haemoglobin, whereby taurine inhibited the formation of advanced glycation end products (AGEs), which plays a key role in development of diabetes complications(238, 239).

A recent review suggests that the inhibition of these reactive oxygen species (ROS) is regulated by mitochondria function with taurine acting as a central role at the level of RNA(240). This lead to the proposal that taurine depletion in conditions such as diabetes may contribute to mitochondrial dysfunction and restoration of taurine level may normalize its function and contribute to inhibition of ROS(9).

In humans, taurine is metabolized into two exchangeable pools, one small rapidly exchanging pool ($T_{1/2}$ 0.1 hours) and a larger slowly exchanging pool ($T_{1/2}$ 70 hours). It is rapidly excreted, predominantly in the urine, 70% as taurine and 25% as sulphate(241). Brons et al measured serum taurine in a randomized crossover study in obese non-diabetic participants, and concluded that 2 weeks washout after each treatment with supplemental taurine or placebo was adequate(242). Moloney et al also have used similar washout period in a crossover study comparing taurine and placebo in patients with T1DM(16).

1.4.2 Taurine, Diabetes and Endothelial Dysfunction

1.4.2.1 Taurine and Animal Models of Diabetes Studies

Initial animal diabetes studies demonstrated benefits of Taurine in suppression of hyperglycaemia, prolonging survival, improving pancreatic cell mass, and improving insulin resistance(9).

In a Streptozocin (STZ)-induced diabetes rats, Taurine supplementation through its antioxidant property demonstrated glycaemic improvement, lowers glycated haemoglobin (HbA_{1c}) and prolongs survival after 6 months therapy, compared to diabetes rats without treatment(243, 244).

In a separate study involving pregnant obese non-diabetic (NOD) mice, Taurine supplementation throughout the gestation, demonstrated greater pancreatic cell mass, reduced rates of insulinitis and delayed the mean onset time for the development of diabetes in the offspring compared to placebo(245).

Similar benefits were also seen in non-insulin dependent obese mice (The Otsuka Long-Evans Tokushima Fatty, OLETF) where Taurine improved hyperglycaemia, insulin resistance, and suppressed serum triglycerides and cholesterol(246, 247).

In metabolic syndrome induced high fructose fed (HFD) mice, Taurine supplementation improved insulin resistance, glucose tolerance, hypercholesterolaemia, hypertriglyceridaemia, total antioxidant capacity, and nitric oxide metabolites by 11 to 56%, suggestive of a protective effect(248).

In similar animal models, Taurine supplementation has been shown to reduce diabetes related complications(9). These include AGE related renal injury and fibrosis (249-251), and diabetes retinopathy(252, 253). Taurine supplementation also reduced oxidation damage in lens and nerves of diabetic animals with potential benefit in preventing cataracts and improvement in neuropathy symptoms(9, 254).

The benefits of Taurine in modulating endothelial dysfunction in diabetes were highlighted in various animal studies. In the Streptozocin induced Type 1 diabetes mouse model, Taurine, through its antioxidant property, has been shown to improve endothelial dysfunction by downregulating LOX-1, an endothelial receptor for ox-LDL, and intracellular adhesion molecule-1 (ICAM-1) expression on aortic vascular endothelium(15). Acute hyperglycaemia seen in diabetes has been shown to activate leucocyte adhesion and migration to endothelium, increased ICAM-1 production and apoptosis of endothelial cells. Rats given supplementations of Taurine prior to exposure to hyperglycaemia had reduced leucocytes action, reduced ICAM-1 production and reduced endothelial cell apoptosis, suggestive of a role for Taurine in protecting against negative consequences of hyperglycaemia(14).

1.4.2.2 Taurine and Diabetes Human Studies

There have been numerous human studies involving supplementation with Taurine in patients with diabetes(9, 16, 255-258). Plasma and platelet taurine levels are lower in participants with type 1 (T1DM) and type 2 diabetes (T2DM) compared to normal subjects(255-257). There's also an inverse correlation between log plasma taurine and glycated haemoglobin (HbA_{1c}) in similar participants(255).

A clinical benefit of taurine in T1DM is still debatable(9). Supplementation of taurine (0.5 g twice daily) in 10 T1DM participants improved carbohydrate metabolism and decreased triglycerides level(259). In contrast, the secondary finding of a study where taurine (1.5g per day) was supplemented in 39 T1DM participants for 90 day abolishes the inverse correlation between log plasma taurine and HbA_{1c} suggestive of no significant effect on glycaemic control. The latter study did show reduction in platelet aggregation and restoration of taurine level similar to control subjects (255).

Taurine supplementation (3g per day) for 4 months in T2DM participants (n=32) did increase plasma taurine level but did not have any significant effect on HbA_{1c} level compared to placebo group(258). In a crossover

study involving overweight, non-diabetic men (n=20), taurine supplementation (1.5g for 8 weeks) had no effect on insulin secretion, insulin sensitivity or on plasma lipid level, suggesting no role of taurine in T2DM prevention in a high-risk group(242).

These studies concluded that taurine supplementation in T2DM does not have any significant effect on glycaemic control, in contrast to prior animal studies(9).

The main limitation to these conclusions is that taurine dose per body weight in animal studies was more than 10 times higher than in human clinical trials. Other limitations include the difference in severity of disease among participants, concomitant use of other medications in human studies and the duration of trials(9).

A crossover clinical study by Xiao et al however, demonstrated usefulness of taurine against the impairment of insulin sensitivity due to increase fatty acids in obese non-diabetic men. By using intravenous infusion of Intralipid, a state of induced insulin resistance was created. Compared to N-acetylcysteine (NAC), 2 weeks pretreatment with taurine ameliorated lipid-induced functional beta cell decompensation and insulin resistance, possibly by reducing oxidative stress(260).

Nakamura et al investigated taurine supplementation (3g/day) on T2DM participants with microalbuminuria for 12 months. Similar to the animal study, taurine supplementation did not show any benefit in improving microalbuminuria and biomarkers of fibrosis such as serum collagen IV and plasma matrix metalloproteinase-9 (261) .

More recently, several studies investigated the role of taurine in endothelial dysfunction. Moloney et al investigated the role of taurine in reversing the endothelial dysfunction in participants with T1DM. Earlier studies by the same group demonstrated reversal of endothelial dysfunction assessed by flow-mediated dilatation (FMD), in diabetics with pravastatin (262) and in young asymptomatic smokers supplemented with taurine(263). In the latter study, they also demonstrated an up-regulation

of nitric oxide from monocyte-endothelial cell interaction in subjects who received taurine.

In their recent crossover study, 9 T1DM participants were randomized to either taurine (1.5g/day) or placebo for 2 weeks each, with assessment of endothelial function using FMD. Control subjects (n=10) underwent a baseline scan only. T1DM participants had significant abnormalities in the measurement of augmentation index and brachial artery reactivity (FMD) indicating early endothelial dysfunction. Both parameters improved after 2 weeks supplementation with taurine suggestive of a potential benefit in preventing progression towards atherosclerosis in this group(16).

1.4.3 Summary

Taurine acts as an antioxidant with proven roles in preventing diabetes and its complications in animal models through various pathways. The benefit in improving glycaemic control in human participants with diabetes is unproven, largely due to different doses used compared to animal study, small sample sizes, concomitant medications, and short duration of study. More recent data suggest a potential role of Taurine in reversing endothelial dysfunction that may benefit patients with diabetes.

2 Study 1: Cardiovascular safety of DPP-4 inhibition in participants with Type 2 Diabetes Mellitus: Endothelial Progenitor Cells as an early marker of long-term cardiovascular risk

2.1 Introduction

Diabetes mellitus is defined as a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defect in insulin secretion, insulin action or both (89). T2DM is characterized by progressive insulin secretory defect on background of insulin resistance. It is a complex chronic illness requiring continuous medical care with multifactorial risk reduction strategies beyond glycaemic control (90).

Today, macrovascular complications (CVD including stroke) are the most common cause of death in participants with diabetes compared to microvascular complications (retinopathy, nephropathy)(90). When diabetes patients develop CVD, they have worse prognosis compared to patients without diabetes(97, 98) and are more likely to develop cardiomyopathy in the future(99, 100).

Endothelial Progenitor Cells (EPCs) are defined as circulating bone marrow derived cells capable of postnatal vasculogenesis and vascular homeostasis (17). Numerous studies have confirmed, both directly and indirectly, the central role of EPCs in cardiovascular repair processes. The accumulated evidences suggests that a balance between the damaging effects of conventional cardiovascular risk factors and the ability of circulating EPCs to effect endothelial repair determines cardiovascular risk(1, 7, 78, 264, 265). EPC numbers in patients with Type 2 Diabetes (T2DM) have been shown to be significantly lower than in non-diabetics, and recent study have shown an increase in EPC numbers with intensive treatment of T2DM(86).

The controversy regarding the potential harmful cardiovascular effects of Rosiglitazone(144) has resulted in an increased awareness of the need to more closely evaluate the potential CV benefits and risks of new

antihyperglycaemic agents(150). Unfortunately, long-term post-marketing trials are often required before an adverse cardiovascular signal can be detected.

Dipeptidyl Peptidase-4 inhibitors (DPP4i) are a new class of treatment for T2DM, which are licensed for use as monotherapy or dual therapy with metformin and/or a sulphonylurea when glycaemic targets are not achieved. DPP-4 degrades glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP); two incretin hormones that have been shown to be important in glucose and appetite regulation. Inhibiting DPP-4 enhances the effect of GLP-1 and GIP, resulting in an increase in glucose-dependent insulin secretion and suppressing post-prandial glucagon secretion. While DPP-4 inhibitors are effective glucose-lowering agents, at the initiation of this study, there were no published data on its cardiovascular safety (155) or benefit.

In a 4 week non-randomized, non-blinded study of EPCs in patients with T2DM, Sitagliptin, a DPP-4 inhibitor was found to elevate the number of EPCs (as determined by flow cytometry) compared to patients prescribed no additional oral anti-diabetic agent(184).

We propose to conduct a long-term open-labeled randomized trial examining the effects of DPP-4 inhibitor (Saxagliptin) versus sulphonylurea therapy on EPC numbers and function in patients with T2DM, in an attempt to find an early marker of potential cardiovascular risk or benefit of these medications in patients with T2DM.

2.2 Methods

Diabetes patients on maximum doses of metformin therapy who require addition of another oral anti-diabetic agent based on clinical need, and in whom the benefits and risks of sulphonylurea (SU) therapy versus DPP-4 inhibitor therapy are felt not to favour one agent over the other, were recruited and randomized to receive either DPP-4 inhibitor (Saxagliptin, SAX) or SU (Gliclazide Modified Release, GLC) treatment.

Participants were identified when they attended the routine visits at the diabetes outpatient clinic and the diabetes day centre at Connolly Hospital from March 2011 to October 2012. The study was discussed and a patient information leaflet was given (Appendix section 4.1.1).

Participants who agreed to the study were given an appointment to attend the diabetes day centre, where fully informed consent (Appendix section 4.1.2) was obtained.

The following inclusion criteria includes having T2DM, HbA_{1c} > 7.0% (53mmol/mol), and on treatment with metformin 850 mg twice a day or more. Participants who were pregnant, on oral contraceptive pill, breastfeeding, had HbA_{1c} > 10% (86mmol/mol), documented renal impairment (cc <60 mL/min) and/or hepatic impairment, alcoholism, had inter-current illness, foot ulcer, significant retinopathy, active infection, previous myocardial infarction, heart failure, stroke, and documented peripheral vascular disease or absent peripheral pulses were excluded from the study.

All participants signed an informed consent and this study was approved by the Connolly Hospital Ethics Committee.

On the first visit, participants had anthropological measurements (weight, body mass index, waist circumference, waist to hip ratio), clinical assessment (medical history, medications, blood pressure), routine blood testing (renal/ liver/ bone profile, HbA_{1c}) and additional blood sampling for EPC culture. Height and weight were measured without shoes and rounded to the closest 0.5cm and nearest kg. Waist circumference was measured using non-stretchable tape, while standing, feet together, midpoint between lower rib margin and iliac crest.

By using a random number generator, each participant was randomly assigned either Saxagliptin 5mg daily or Gliclazide MR 30mg daily as a second line anti-diabetic treatment. Participants were contacted monthly in order to monitor any potential side effects. Participants in the Gliclazide group had their doses titrated to a maximum of 120mg daily based on blood glucose diary review. Dose of Gliclazide was reduced if there was

any evidence of hypoglycaemia. Participants on Saxagliptin remained on the same dose throughout the study.

Anthropometric measurements, clinical assessment, blood testing and EPC culture were repeated at 6 months review. At that time, participant's overall diabetic control was reviewed and further treatment recommendations were planned as per the current treatment guidelines.

2.2.1 EPC Isolation and Adhesion Assay

2.2.1.1 EPC Isolation

The technique for EPC isolation was similar to previous studies in our group(4, 39, 266). 60 mls of peripheral blood was decanted into 3 x 20mls of Hanks Balanced Salt Solution (HBSS). This mixture was layered onto 4 x 15 mls of Ficoll-Paque giving to a total of 45mls for each bottle.

Each sample was centrifuged at 1800 rpm at 30 minutes with acceleration of 3 and deceleration of 0. By using a Pasteur pipette, the mononuclear cell layer was separated from each bottle into another 50mls bottle.

The removed mononuclear layer was centrifuged at 1600 rpm for 10 minutes with acceleration of 3 and deceleration of 0. 5mls of Red Cell Lysis buffer was added to the pellet, which then undergoes another centrifugation at 1600 rpm for 10 minutes at acceleration and deceleration of 9.

This process was repeated twice, with further pellet washing using Phosphate Buffer Saline (PBS) before suspending the pellet in Endothelial Growth Media 2 (EGM-2) solution.

Using a haemocytometer, the volume of cells corresponding to 10×10^6 were calculated. This volume constitutes the amount of cells to be plated into each well of fibronectin coated 6-well plates. The desired volume of cells was added onto the corresponding volume of EGM-2 to give a total volume of 2000 μ l for each well of the 6-well plate.

The plate was labeled and incubated in a 37⁰c with 5% of CO₂ incubator for 7 days. On day 4, the non-adherent cells were removed and the media was replaced.

2.2.2 Identification of EPC

On day 7, after washing the well, 10µl of acetylated low-density DiL-labelled LDL (DiL-AcLDL) were added followed by 1ml of media (Figure 2-1).

After 4 hours incubation, cells were washed and media was replaced. By using fluorescent microscope and red filter, cells incorporating DiL-AcLDL fluorescence red were identified as potential circulating EPCs (Figure 2-2).

20mcl of *Ulex* Lectin were added to the well. After 3 hours of incubation, cells were washed and media was replaced. Using the fluorescent microscope and green filter, cells expressing Lectin, with fluorescence green was identified as potential EPCs (Figure 2-3).

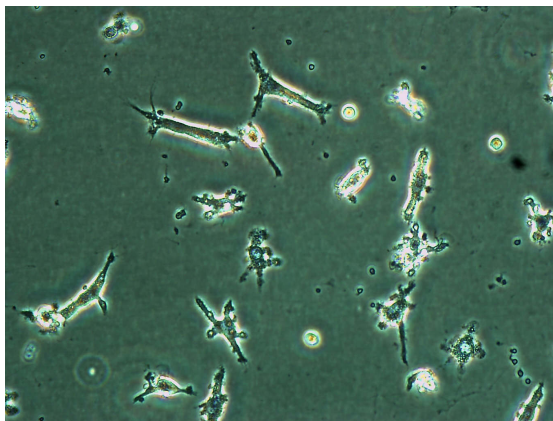


Figure 2-1: Dual staining of Endothelial Progenitor Cells (EPCs) at day 7 under light microscopy (400x magnification).

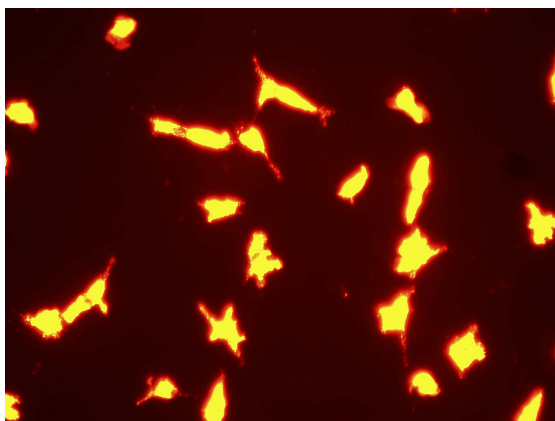


Figure 2-2 Dual staining of EPCs. Incorporation of acetylated low-density DiI-labelled LDL (DiI-AcLDL) (red colour) under fluorescence microscope with red filter

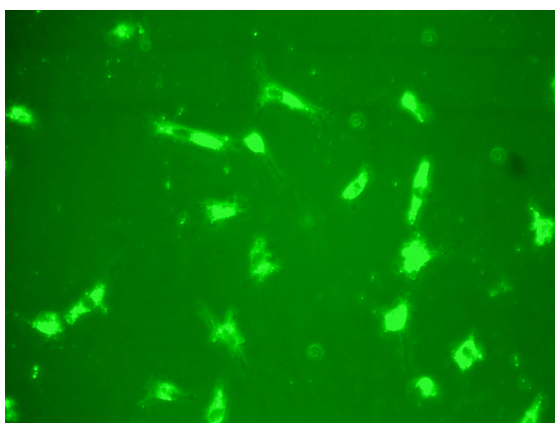


Figure 2-3: Dual staining of EPCs. Incorporation of *Ulex* Lectin (green colour) under fluorescence microscope with green filter

2.2.3 Counting EPC

On day 7, the 6-well plates are washed twice to remove any non-adherent cells. Adherent cells in 12 random microscope fields (4 in each randomly chosen well) were photographed at 400x magnification before another period of incubation for 15 minutes. Cells in the photographed were counted by a single investigator and was validated by an independent investigator.

After 15 minutes, Enzyme Free Cell Dissociation Buffer was added to each well followed by 30 minutes of incubation. Detached cells underwent centrifugation at 1500 rpm for 10 minutes.

Pellet was suspended in Serum Free Media (SFM) and using a haemocytometer, the volume of cells corresponding to 5×10^4 cells were calculated.

2.2.4 Adhesion Assay

Using a 96-well plate, Human Fibronectin 0.1% and HBSS were added to wells A1 to A5 and control wells (H1 to H5). After an overnight incubation, these wells were blocked using Bovine Serum Albumin (BSA) and incubated for 2 hours.

After 2 hours, each well was washed using PBS.

Detached day 7 cells (5×10^4) were plated onto wells A1 to A5. A similar volume of SFM without cells was added onto control wells before they were incubated for 1 hour.

After 1 hour, wells were washed with PBS and cells were fixed with formaldehyde.

Crystal violet was added to each well and after rinsing with PBS, plates were allowed to dry for 30 minutes.

The centre of each well with cells was photographed (Figure 2-4).

Methanol was added to wells to elute the stains from the cells before undergoing analysis in the plate reader at 595nm wavelength for 0.1sec.

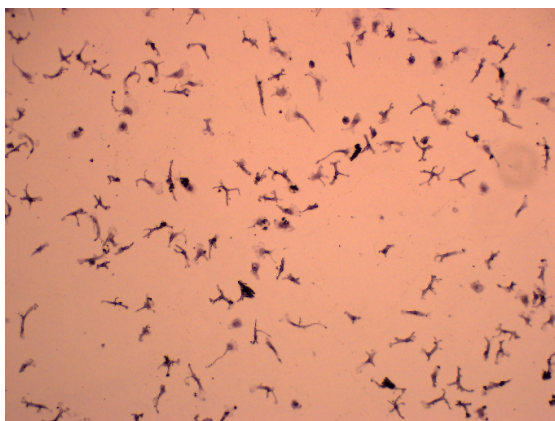


Figure 2-4: EPC Adhesion assay. Isolated EPC stained with crystal violet (purple colour) adhering to fibronectin coated well on Day 7.

2.3 Statistical analysis

Data were analyzed based on treatment group. Categorical data were compared using chi-square test. Continuous data were tested for normality. Normally distributed data were expressed using mean \pm SD and non-normally distributed data were expressed in median (IQ range). Independent sample T-test was used in normally distributed data while Mann-Whitney test was used in non-normally distributed data when comparing between treatment groups. Eta squared was used to calculate the magnitude of the differences in means between treatment groups. Spearman correlation was used to correlate parameters at baseline. Paired T-test and Wilcoxon Signed Rank Test was used to compare data at baseline and at 6 months follow up based on randomization. Significant p value of < 0.05 is considered significant in all data analyses.

2.4 Results

A total of 18 participants with T2DM on metformin monotherapy requiring diabetes intensification were identified, 12 were males and 6 were females.

Baseline age, body mass index (BMI), waist hip ratio (WHR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) are illustrated in Table 2-1. Mean diabetes duration was 4 years with a baseline glycated haemoglobin (HbA_{1c}) of 58.7 ± 8.6 mmol/mol. Baseline creatinine and spot urine albumin creatinine ration (ACR) were 73.3 ± 19.2 mmol/L and 0.85 (0.55 – 2.7) mg/mmol respectively. Baseline total cholesterol (TC), HDL cholesterol (HDL), LDL cholesterol (LDL) and Triglycerides (TG) were 4.2 ± 1.1 , 1.1 (1.1 – 1.3), 2.2 ± 0.8 and 1.8 ± 1.1 mmol/L respectively. Baseline EPC count was 30 (21 – 43) cells/hpf while EPC adhesion capacity was 0.24 (0.16 – 0.69) fluorescence unit respectively.

Table 2-1 Parameters at baseline (Study 1). Normally distributed data are expressed as mean \pm SD while skewed data are expressed as median (interquartile range, IQ range). ACEI (Angiotensin Converting Enzyme Inhibitor), ARB (Angiotensin Receptor Blocker), BMI (Body Mass Index), HbA_{1c} (Glycated Haemoglobin), ACR (Albumin Creatinine Ratio), HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), EPC (Endothelial Progenitor Cells)

Baseline Characteristics	Values
Total patients	18
Age (years)	56.5 \pm 7.6
Diabetes Duration (years)	4.11 \pm 2.3
Gender (n, %)	
Male	12 (66.7)
Female	6 (33.3)
BMI (kg/m ²)	30.2 \pm 2.9
Waist Hip Ratio (WHR)	0.96 \pm 0.07
Systolic Blood Pressure (mmHg)	141.7 \pm 22.7
Diastolic Blood Pressure (mmHg)	85.7 \pm 12.1
Concomitant Medications (n, %)	
Statin	16 (88.9)
Aspirin	17 (94.4)
ACEI	5 (27.8)
ARB	4 (22.2)
Metformin	18 (100)
Smoker (n, %)	3 (16.7)
Fasting Glucose (mmol/L)	8.3 \pm 1.5
HbA _{1c} (mmol/mol)	58.7 \pm 8.6
Creatinine (mmol/L)	73.3 \pm 19.2
Urine ACR (mg/mmol)	0.85 (0.55 – 2.7)
Total Cholesterol (mmol/L)	4.2 \pm 1.1

Baseline Characteristics	Values
HDL Cholesterol (mmol/L)	1.2 (1.1 – 1.3)
LDL Cholesterol (mmol/L)	2.2 ± 0.8
Triglycerides (mmol/L)	1.8 ± 1.1
EPC per high power field (EPC/hpf, cells)	30 (21 – 43)
EPC adhesion (fluorescence unit)	0.24 (0.16 – 0.69)

Correlation analysis was performed to identify any association between EPC and parameters at baseline. There were positive correlations between diabetes duration and the numbers of EPC per high power field (EPC/hpf, $\rho = 0.522$, $p=0.032$) and EPC adhesion capacity (EPC adhesion, $\rho = 0.518$, $p=0.028$) (Figure 2-5 and 2-6). Baseline diastolic blood pressure (DBP) however had an inverse relationship to EPC adhesion ($\rho = -0.52$, $p=0.026$) (Figure 2-7). There were no significant correlations between baseline EPC/hpf or EPC adhesion to age, gender, smoking status, concomitant treatment [statin, Angiotensin Receptor Blocker (ARB), Angiotensin Converting Enzyme Inhibitor (ACEI), and aspirin], WHR, glycaemic control and lipid levels.

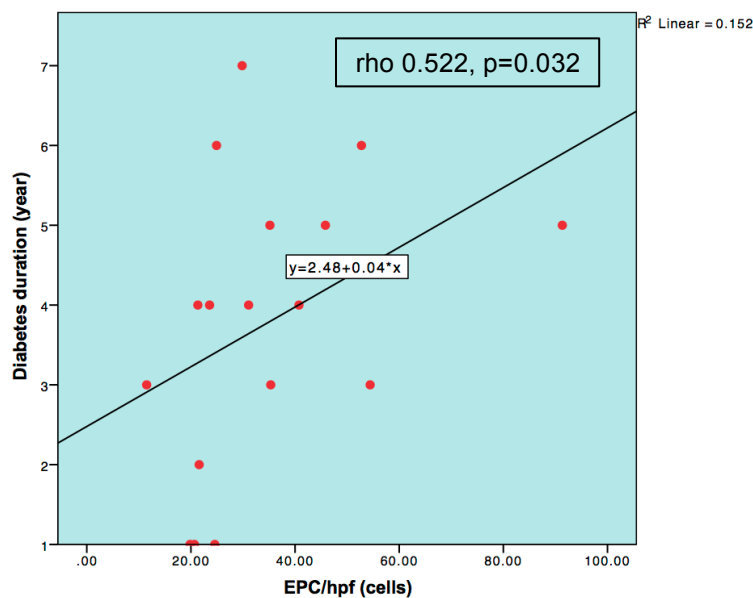


Figure 2-5 Spearman correlation between diabetes duration and EPC numbers at baseline (Study 1). There was a positive relationship between the numbers of EPC per high power field (EPC/hpf, cell) and diabetes duration (years) at baseline.

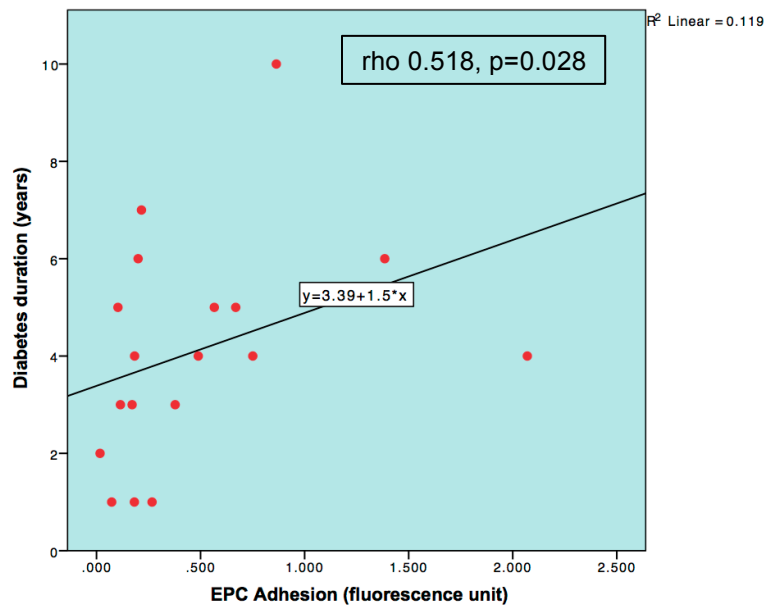


Figure 2-6 Spearman correlation between diabetes duration and EPC adhesion at baseline (Study 1). There was a positive relationship between EPC Adhesion (fluorescence unit) and diabetes duration (years) at baseline.

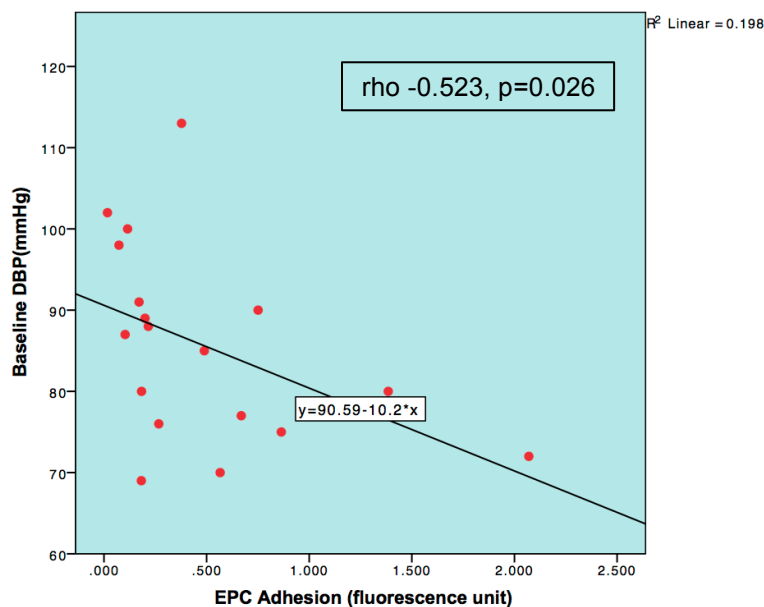


Figure 2-7 Spearman correlation between baseline DBP and EPC adhesion (Study 1). There was a negative relationship between EPC Adhesion (fluorescence unit) and diastolic blood pressure (DBP, mmHg) at baseline.

Participants were randomized to either SAX (n=7) or GLC (n=11) as part of their treatment intensification. Due to technical reasons, one participant did not have adequate EPC cells counted at baseline while another participant did not have adequate EPC cell culture at 6 months review.

There were no significant differences of age, gender, smoking status, BMI, WHR, SBP, DBP, diabetes duration, and glucose at baseline between treatment groups (Table 2-2). There were no significant differences in the concomitant use of statin, ARB, ACEI or aspirin between treatment groups. There were no significant differences in the number of total EPC/hpf with median (IQ range) of 33 (19 – 44) cells in SAX group (n=6) and 25 (22 – 46) cells in the GLC group (n=11, p=0.9). Similarly, there were no differences in the adhesion capacity of EPC between both groups with median (IQ range) of 0.22 (0.17 – 0.86) fluorescence units in the SAX group (n=7) compared to 0.27 (0.1 – 0.67) fluorescence units in the GLC group (n=11, p=0.75).

At baseline, HbA_{1c} was higher in the GLC group compared to SAX group (62.3 ± 8.8 vs. 53.1 ± 4.4 mmol/mol, p=0.02) while creatinine level was higher in SAX group compared to GLC group (88.9 ± 15.2 vs 63.5 ± 14.5 mmol/L, p=0.003). The magnitude of the differences between the means of HbA_{1c} and creatinine were large with an eta squared of 0.44 for HbA_{1c} and 0.29 for creatinine respectively.

Table 2-2 Comparison between baseline parameters based on treatment (Study 1). HbA_{1c} and Creatinine levels were different at baseline. Normally distributed data are expressed as mean \pm SD while skewed data are expressed as median (interquartile range, IQ range). BMI (Body Mass Index), WHR (Waist Hip Ratio), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), HbA_{1c} (Glycated Haemoglobin), ARB (Angiotensin Receptor Blocker), ACEI (Angiotensin Converting Enzyme Inhibitor), ACR (Albumin Creatinine Ratio). HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), EPC (Endothelial Progenitor Cells). Significant differences considered at $p < 0.05$.

Baseline Parameters	Saxagliptin (n=7)	DMR (n=11)	Sig
Gender n (%)			
Female	1(14.3)	5(45.5)	0.39
Male	6(85.7)	6(54.5)	
Smoker n (%)	0	3(27.3)	0.39
Age (mean \pm SD, years)	57.9 \pm 6.1	55.5 \pm 8.6	0.54
BMI (mean \pm SD, kg/m ²)	29.4 \pm 1.8	30.7 \pm 3.4	0.36
WHR n (mean \pm SD)	6(0.94 \pm 0.33)	11(0.97 \pm 0.08)	0.44
SBP (mean \pm SD, mmHg)	145.1 \pm 28.1	139.5 \pm 19.7	0.62
DBP (mean \pm SD, mmHg)	88.4 \pm 14.5	83.9 \pm 10.7	0.46
Diabetes Duration n (mean \pm SD, years)	4.9 \pm 2.7	3.6 \pm 2.0	0.29
Creatinine (mean \pm SD, mmol/L)	88.9 \pm 15.2	63.5 \pm 14.5	0.003

Baseline Parameters	Saxagliptin (n=7)	DMR (n=11)	Sig
Urine ACR, n [med(IQ range), mg/mmol]	6, 0.85 (0.55 – 10.38)	11, 0.85 (0.5 – 2.5)	0.61
Glucose, n (mean \pm SD, mmol/L)	5(7.5 \pm 0.7)	7(8.8 \pm 1.8)	0.16
HbA1c (mean \pm SD, mmol/mol)	53.1 \pm 4.4	62.3 \pm 8.8	0.02
Total Cholesterol (mean \pm SD, mmol/L)	3.7 \pm 1.0	4.6 \pm 1.1	0.09
HDL Cholesterol [med(IQ range), mmol/L]	1.2 (1.0 – 1.6)	1.2 (1.1 – 1.3)	0.82
LDL Cholesterol (mean \pm SD, mmol/L)	1.8 \pm 0.7	2.4 \pm 0.7	0.12
Triglycerides (mmol/L)	1.3 \pm 0.6	2.0 \pm 1.2	0.18
Medications n (%)			
Statin	6 (85.7)	10 (90.9)	1.0
ARB	3 (42.9)	1 (9.1)	0.27
ACEI	4 (57.1)	5 (45.5)	1.0
Aspirin	7 (100)	10 (90.9)	1.0
Total EPC per HPF, n [med(IQ range), cells/hpf]	6 [33 (19 – 44)]	11 [25 (22 – 46)]	0.9
EPC Adhesion, [med(IQ range), fluorescence unit]	7[0.22(0.17 – 0.86)]	10[0.27 (0.1 – 0.67)]	0.75

In all participants, the addition of a second antihyperglycaemic medication improved HbA_{1c} at 6 months compared to baseline (54.9 ± 8.4 vs. 58.9 ± 8.8 mmol/mol, $p=0.06$).

Table 2-3 demonstrates the comparison of all parameters at baseline and at 6 months, based on randomized intervention. In the GLC group, there was a downward trend of HbA_{1c} from 62.3 ± 8.8 to 57.1 ± 8.5 mmol/mol ($p=0.08$) and from 53.1 ± 4.4 to 50.4 ± 6.6 mmol/mol ($p=0.33$) in the SAX group. Fasting glucose were significantly lower in GLC group (9.1 ± 1.8 to 7.9 ± 1.8 mmol/L, $p=0.02$) while no difference seen in SAX group (7.4 ± 0.7 to 7.2 ± 1.4 mmol/L, $p=0.73$) (Table 2-3, Figure 2-8). Weight was significantly increased in the GLC group at 6 months (85.96 ± 14.68 to 87.89 ± 14.93 kg, $p=0.008$) while no change was seen in SAX group (84.23 ± 9.9 to 84.51 ± 9.55 kg, $p=0.75$). This contributed to the increased of BMI in GLC group (30.7 ± 3.4 to 31.4 ± 3.4 kg/m², $p=0.02$) compared to SAX group (29.4 ± 1.8 to 29.2 ± 2.0 kg/m², $p=0.72$) (Table 2-3, Figure 2-9). There were no significant changes in SBP, DBP, cholesterol profile, and urine ACR in each group, during follow up. All smokers ($n=3$ in the GLC group) reported continuing smoking during the study.

Table 2-3: Comparison between changes in parameters with treatment (Study 1). BMI and fasting glucose were different at follow up in GLC group. BMI (Body Mass Index), WHR (Waist Hip Ratio), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), HbA1C (Glycated Haemoglobin), ARB (Angiotensin Receptor Blocker), ACEI (Angiotensin Converting Enzyme Inhibitor), ACR (Albumin Creatinine Ratio), SAX (Saxagliptin), GLC (Gliclazide MR), HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), EPC (Endothelial Progenitor Cells). Significant value $p < 0.05$.

Parameters	Intervention	Baseline	6 months	Sig
BMI, n (mean \pm SD, kg/m ²)	SAX	7(29.4 \pm 1.8)	7(29.2 \pm 2.0)	0.72
	GLC	11(30.7 \pm 3.4)	11(31.4 \pm 3.4)	0.02
WHR, n (mean \pm SD)	SAX	6(0.94 \pm 0.03)	6(0.93 \pm 0.03)	0.24
	GLC	11(0.97 \pm 0.08)	11(0.95 \pm 0.08)	0.33
SBP, n (mean \pm SD, mmHg)	SAX	7(145.1 \pm 28.1)	7(146.1 \pm 19.4)	0.82
	GLC	11(139.5 \pm 19.7)	11(138.5 \pm 23.4)	0.87
DBP, n (mean \pm SD, mmHg)	SAX	7(88.4 \pm 14.5)	7(89 \pm 7.9)	0.85
	GLC	11(83.9 \pm 10.7)	11(81.5 \pm 11.1)	0.35
Creatinine, n (mean \pm SD, mmol/L)	SAX	7(88.9 \pm 15.2)	7(87 \pm 13.7)	0.16
	GLC	11(63.5 \pm 14.5)	11(66.1 \pm 15.5)	0.23
Glucose, n (mean \pm SD, mmol/L)	SAX	4(7.4 \pm 0.7)	4(7.2 \pm 1.4)	0.73
	GLC	6(9.1 \pm 1.8)	6(7.9 \pm 1.8)	0.02

Parameters	Intervention	Baseline	6 months	Sig
HbA _{1c} , n (mean \pm SD, mmol/mol)	SAX	7(53.1 \pm 4.4)	7(50.4 \pm 6.6)	0.33
	GLC	11(62.3 \pm 8.8)	11(57.1 \pm 8.5)	0.08
Urine ACR, n [med(IQ range), mg/mmol]	SAX	6[0.85 (0.55 – 10.4)]	7[1.0(0.5 – 10.5)]	0.67
	GLC	11[0.85(0.5 – 2.5)]	10[0.7(0.48 – 1.48)]	0.8
Total Cholesterol, n (mmol/L)	SAX	7(3.7 \pm 1.0)	7(3.7 \pm 0.7)	0.92
	GLC	11(4.6 \pm 1.1)	11(4.5 \pm 1.3)	0.6
HDL Cholesterol, n [med(IQ range), mmol/L]	SAX	7[1.2(1.0 - 1.6)]	7[1.4(1.1 – 1.7)]	0.55
	GLC	11[1.2(1.1 – 1.3)]	11[1.2(1.1 – 1.3)]	0.76
LDL Cholesterol, n (mmol/L)	SAX	7(1.8 \pm 0.7)	7(1.6 \pm 0.5)	0.24
	GLC	10(2.4 \pm 0.8)	10(2.3 \pm 1.0)	0.93
Triglycerides, n (mmol/L)	SAX	7(1.3 \pm 0.6)	7(1.3 \pm 0.5)	0.7
	GLC	11(2.0 \pm 1.2)	11(2.0 \pm 1.3)	0.77
Total EPC/hpf, n [med(IQ range), cells/hpf]	SAX	6[33(19 – 44)]	7[35(28 – 38)]	0.92
	GLC	11[25(22- 46)]	10[39(28 – 46)]	0.24
EPC Adhesion, n [med(IQ range), fluorescence unit]	SAX	7[0.22(0.17 – 0.86)]	7[0.3(0.17 – 0.65)]	1.0
	GLC	11[0.27(0.1 – 0.67)]	10[0.32(0.14 – 0.84)]	0.88

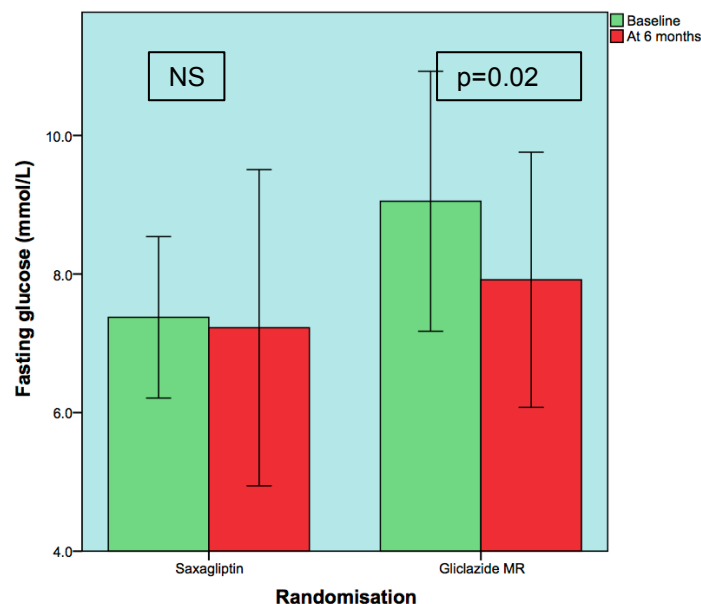


Figure 2-8 Changes in mean fasting glucose (mmol/L) at 6 months compared to baseline according to treatment (Study 1). Fasting glucose was lower in Glucoside MR (GLC) group while no changes seen in Saxagliptin group. Error bar represents confidence interval at 95%. NS (Non significant). Significant difference at $p < 0.05$.

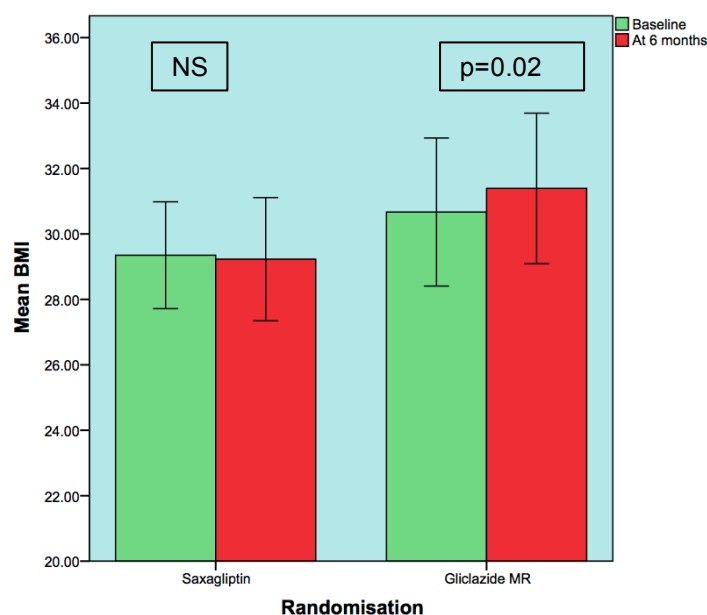


Figure 2-9 Changes in mean BMI (kg/m²) at 6 months compared to baseline according to treatment (Study 1). BMI was higher in Glucoside MR (GLC) group while no changes seen in Saxagliptin group. Error bar represents confidence interval at 95%. NS (Non significant). Significant difference at $p < 0.05$.

In the SAX group, there were no significant changes in EPC numbers per high power field at 6 months compared to baseline [n=7, 35 (28 – 38) from n=6, 33 (19 – 44) cells/hpf, p=0.9] (Figure 2-10). Similarly, there were no significant changes in EPC adhesion capacity at 6 months compared to baseline [n=7, 0.3 (0.17 – 0.65) from n=7, 0.22 (0.17 – 0.86) fluorescence units, p=1.0] (Figure 2-11).

In the GLC group, no significant changes were demonstrated in the EPC numbers per high power field at 6 months [n=10, 39 (28 – 46) from n=11, 25 (22 – 46) cells/hpf, p=0.24] (Figure 2-10) and EPC adhesion capacity [n=10, 0.32 (0.14 – 0.84) from n=11, 0.27 (0.1 – 0.67) fluorescence units, p=0.88] compared to baseline (Figure 2-11).

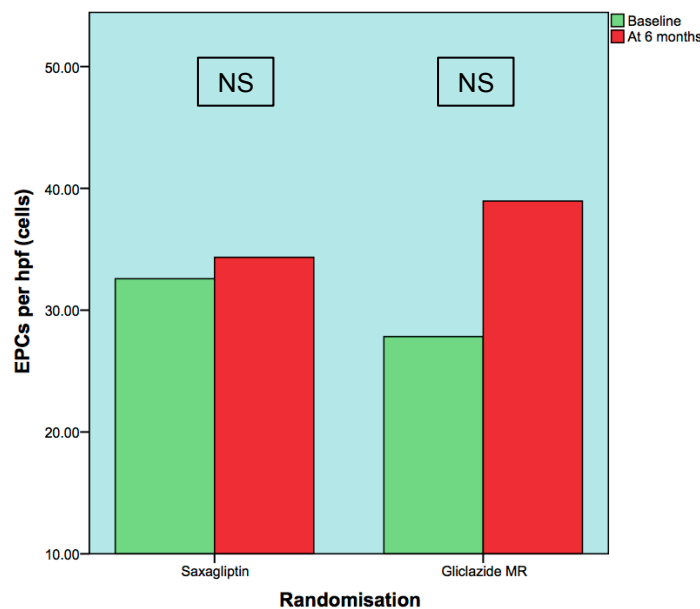


Figure 2-10 Changes in median EPC numbers per high power fields (hpf) at 6 months compared to baseline according to treatment (Study 1). No difference between EPC/hpf at 6 months in both groups compared to baseline. NS (Non significant)

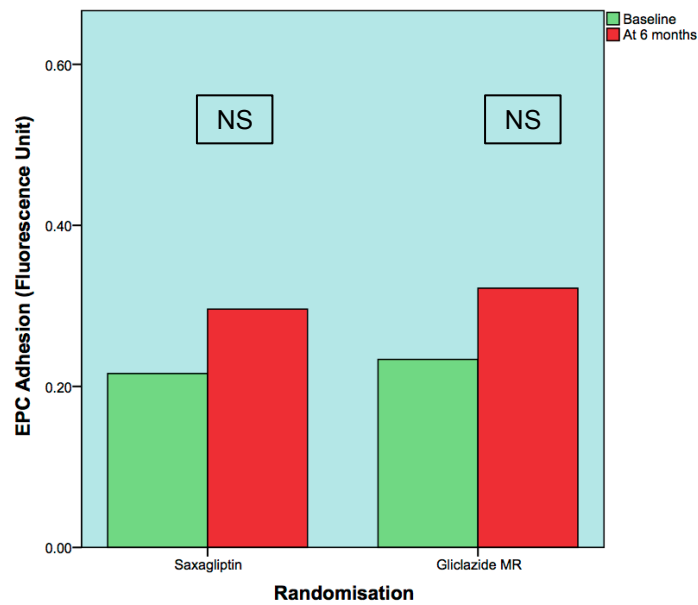


Figure 2-11: Changes in median EPC adhesion at 6 months compared to baseline according to treatment (Study 1). No difference between EPC adhesion (fluorescence unit) at 6 months in both groups compared to baseline. NS (Non significant).

2.5 Discussion

We demonstrated no significant difference in EPC numbers and adhesion capacity in participants with T2DM treated for 6 months with either Saxagliptin or Gliclazide MR in addition to Metformin therapy. The result may be interpreted as an absence of early adverse vascular changes when Saxagliptin is added to Metformin therapy, and a potential neutral CV outcome profile, compared to Gliclazide MR, a well-established glucose-lowering medication, in participants with T2DM.

EPC is a surrogate biological marker for vascular function and cumulative vascular risk. It has an inverse correlation with the traditional CV risk assessment such as Framingham score and cholesterol level with a positive correlation with flow mediated dilatation (FMD)(7); a well validated measurement of arterial stiffness(196). EPC changes early in vascular disease and have been shown to predict atherosclerosis progression, as demonstrated in a 10-year prospective study involving healthy controls and participants with coronary artery disease and acute coronary syndrome(78). When our study was performed, there was no published long-term CV safety data on DPP4i especially Saxagliptin. As a surrogate marker of future CVD, any changes in EPC while on this treatment may influence treatment decision for patients who require intensification of therapy.

We choose a Sulphonylurea (SU) as our comparator as it is widely used, and are considered safe. Gliclazide have been shown to have a free radical scavenging activity due to its unique aminoazabicyclo-octane ring resulting in an improvement in EPC numbers in patients with T2DM better than metformin monotherapy alone(187). This may contribute the slight improvement seen in EPC in the GLC group in our study, although not reaching statistical significance.

2.5.1 Comparison to other published studies

Studies in T2DM patients, evaluating the effects of DPP4 inhibitors on EPC numbers and functions have been performed by Poncina (185) and Fadini et al(184) using Saxagliptin and Sitagliptin respectively. Both of

these studies used different methodology compared to ours. However, their findings can provided some insight to our results.

In the study by Fadini et al, the addition of Sitagliptin for 4 weeks improved the level of EPC by 2 fold and plasma SDF1- α by 50%, in T2DM patients already treated with metformin and/or secretagogues when compared to no treatment(184).

Compared to our study, participants in this study were older and had higher CV risk with preexisting micro and macrovascular complications. Subjects had shorter follow up period of 4 weeks and control patients were not assigned to any additional treatment during follow-up.

EPCs were measured using flow cytometry and without any additional cell functional study. In our study, by assigning each participant with either Gliclazide MR or Saxagliptin for a longer period of 6 months, we were able to compare the newer class of antidiabetic medication to a well-recognized treatment with a proven CV safety profile. By performing EPC culture and adhesion study, we were able to compare not only the changes in numbers but also the function of EPCs, and characterize any potential CV benefit or harm.

Our study methodology and patient selection criteria are more clinically relevant in terms of helping clinicians to decide in a real world setting the benefit/risk of new antidiabetic medications for patients who needed optimization of their diabetes control.

The increasingly important role of SDF1- α was highlighted by Poncina et al. Saxagliptin at an equivalent dose of 5mg and/or SDF1- α was added to the cell culture of pro-angiogenic cells (PACs) including EPC, derived from patients with T2DM(185).

In parallel with our results, in this study, Saxagliptin and/or SDF1- α did not restore the formation of PACs colony and had no effect on the percentage of cells with dual-staining acLDL⁺Lectin⁺, which are cells of interest to us.

Saxagliptin in the presence of SDF1- α however, increased PACs cell adhesion in T2DM patients while it has no effect on PACs from healthy volunteers. T2DM patients had lower genes expressions responsible for regulating cell cycles, apoptosis, and adhesions. Additionally, there was higher expression of DPP4 gene, which further reduces the availability of SDF1- α .

As we did not measure the level of SDF1- α in our study, we can only postulate that the level would be similarly attenuated in our cohort. The inability of Saxagliptin to modify this gene expression may explain the absent of effect seen in our study.

Similar to the study by Fadini et al, subjects were older in the Poncina study and had higher rates of vascular complications at baseline compared to our cohort. Saxagliptin was added to the in-vitro assay rather than as part of the treatment for diabetes. Only 1/3 of the PACs cells in this study double stained positive for acetylated LDL and Ulex-lectin (acLDL⁺ Lectin⁺) and the heterogeneous nature of PACs cultures may potentially be a limiting factor into the interpretation of the findings (185). For EPC adhesion study, we used matrix molecule fibronectin instead of HUVEC as a medium for adhesion and this has been reliably validated as a suitable medium for EPC adhesions(2, 4).

2.5.2 Discussion of Literature in Relation to Study Results

We identified a positive correlation between the numbers of EPCs and EPC adhesion capacity and the duration of diabetes at baseline in our cohort. We also demonstrated a negative correlation between DBP and EPC adhesion at baseline. The mean diabetes duration at baseline in our participants was 4.11 ± 2.3 years. A previous cross sectional study of 425 subjects with normal glucose tolerance, pre-diabetes, and T2DM demonstrated 2 nadir points of EPC as measured using flow cytometry. Adjusted for any potential confounders, the 1st nadir point happens at diagnosis, followed by a maximum partial recovery at ≤ 10 years post diagnosis before a second nadir at 20 years post diagnosis of diabetes(170). It is possible that the levels of EPCs in our participants

were in the recovery phase at the time of participation in our study and this may also contribute to the positive effect seen on the DBP. At the same time, we could not rule out a potential type-2 error contributing to the findings, considering a small sample size.

HbA_{1c} level was significantly higher in GLC group at baseline compared to SAX group. Although the difference was large (as per eta squared), we demonstrated no significant correlation between HbA_{1c} level and EPC numbers at baseline. Additionally, there are multifactorial factors contributing to rise and fall of EPC (38, 85, 87, 267) rather than HbA_{1c} alone. In the intervention study by Fadini et al, a negative correlation between EPC numbers and fasting glucose were demonstrated at baseline. However, the correlation was lost 4 weeks into treatment with Sitagliptin, despite improvement of plasma glucose and EPC level(184). This supports the notion that many other factors contribute to EPC improvement rather than glycaemic improvement alone.

Due to their higher baseline HbA_{1c}, it is not surprising that participants in the GLC group had a statistically significant improvement in fasting glucose and demonstrated a trend of improvement in HbA_{1c} compared to the SAX group. Due to the difference in mode of action, we were not surprised to find that BMI was significantly elevated in GLC group while SAX group demonstrated no weight changes (weight neutral). This, together with the clinical improvement in HbA_{1c}, suggests that our participants were treated adequately and at least were compliant with their treatment.

Our results parallel the most recent Saxagliptin CV outcome study, the SAVOR-TIMI 53, which demonstrated no increased risk of primary or secondary CV composite end-points including in patients with pre-existing heart failure(155). Unexpectedly, there was an increase in hospitalization for heart failure (CHF) in patients randomized to Saxagliptin compared to placebo. The risk was higher in patients with high overall risk of heart failure (pre-existing disease, impaired renal function and elevated baseline NT-proBNP) and considerably lower in patients with low risk at baseline. Considering the study as one of the largest studies involving

diabetes patients with pre-existing heart failure (n=2105), the absence of pre-clinical data or known mechanisms causing heart failure due to DPP4 inhibition and the nature of this study with multiple testing, this unexpected finding remained unexplained(268).

The fact that the results of our study are consistent with an outcome of a large long-term clinical outcome study, it continue to add to the evidence that EPC are useful early markers of long-term CV risk in the evaluation of any therapeutic agent.

2.5.3 Limitations and Strengths

Our study has a few limitations. Despite our best effort, we managed to only recruit 18 participants within 18 months from our diabetes clinic and diabetes day centre. With more subjects, we may be able to provide additional power to our analysis, minimizing the risk of statistical error. At baseline, our participants had a short duration of diabetes, most were on multidrug therapy, and potentially therefore had lower risk for CVD compared to other studies. As highlighted, this may contribute to a natural improvement of EPC beyond glycaemic influence. It is not known if this natural recovery at baseline reached a threshold for maximum reduction in CV risk. Future study involving comparison of EPC with normal healthy control may answer this. However, we felt that the criteria in selecting the study population are justified. As Saxagliptin is being used more often as a second line agent, in patients relatively early in the diagnosis of diabetes, the impact of this finding will affect this cohort more than other diabetics with higher CV risk and longer duration.

We kept our study as close to the real world experience as much as possible. Our participants were recruited on the basis of clinical need, in whom the benefits and risks of Gliclazide MR versus Saxagliptin were felt not to favour one agent over the other. Rather than using to placebo, we compared a new class of antidiabetic to a well-known antidiabetic medication with proven long-term use without CV complications. Participants were treated and followed up as per standard current practice. These steps are important in ensuring that our results are more

clinically relevant, hoping that we may guide clinicians to tailor their treatment to their patient.

In order to further evaluate the risk/benefit of DPP4i, future studies should include more participants with T2DM with healthy controls as a comparator. A different cohort with a stable history of CVD or CHF is the natural next step.

2.5.4 Summary

In summary, EPCs are well-established surrogate markers of CVD in general and diabetes population. Establishing CV safety of new antidiabetic medication is paramount. Therefore, finding an early markers of CV risk are important. We demonstrated no differences in EPC numbers and function between a DPP4i and a well-established antidiabetes medication. Our results echo the clinical data from trials published after our research was begun supporting a neutral CVD profile of Saxagliptin as an adjunct to the treatment of T2DM.

The preliminary results of this study were published as a poster publication in the International Congress of Endocrinology/ENDO (ICE/ENDO) 2014, Chicago, USA, June 2014 (Poster MON-1051) and the Irish Endocrine Society (IES) 39th Annual Meeting 14th – 15th November 2014 (POSTER 3) (Appendix section 4.1.3).

3 Study 2: The Effect of Taurine on Endothelial Dysfunction In Type 2 Diabetes Mellitus: A Pilot Study

3.1 Introduction

There is a two- to three-fold increase in cardiovascular (CVD) mortality in T2DM patients compared to aged-matched non-diabetic subjects(5). Addressing coexisting risk factors such as hypertension, hypercholesterolaemia and obesity are cornerstones in reducing CVD mortality(109).

The earliest detectable finding in the pathogenesis of atherosclerosis is endothelial dysfunction(5). Cardiovascular risk factors contribute to endothelial injury, disrupting the balance between nitric oxide (NO) and potent vasoconstrictor oxidants, resulting in the inability to maintain vascular tone and reactivity(269). This stiff artery increases ventricular pressure and further attenuates NO production and promotes more atheromatous plaque production(196). Arterial stiffness has been shown to be a strong independent predictor of future CV events and all cause mortality in general population(6).

Taurine is a sulfur-containing amino acid, synthesized in the body in limited amounts, but also present in food, with largest quantities in seafood(8, 9). It has been shown to have a beneficial effect in hypertension(10, 11), hypercholesterolaemia(12, 13), the rate of progression of diabetic nephropathy(249) and endothelial function in animal models of diabetes(14, 15). Potential relevance to human disease was shown by the WHO-CARDIAC Study of taurine levels in a 24-population sample worldwide. The Japanese population in this study had higher taurine levels than the Chinese, European, North American and Oceanic Caucasians. This cross-sectional study demonstrated for the first time, an independent inverse association between population levels of taurine excretion and ischaemic heart disease (IHD) mortality(8).

Taurine exerts its effect through its antioxidant properties. It reacts to the hydrogen peroxide-myeloperoxidase-chloride (HClO) system,

reduces the oxidative damage by LDL, regulates mitochondrial function, inhibits reactive oxygen species (ROS) and further increases the bioavailability of nitric oxide (NO)(9, 233, 240). Studies in diabetes have shown that taurine levels are lower in diabetes patients compared to non-diabetic controls(255-257). Furthermore, diabetes and its complications are strongly associated with the excess of oxidative stress, reduced mobilization of endothelial progenitor cells (EPC) and vascular damage(172).

Endothelial dysfunction is also associated with reduced numbers of EPCs(7). EPCs are circulating bone marrow derived cells, which have the ability to promote vasculogenesis and repair damaged blood vessels(17). All risk factors for atherosclerosis have been associated with reduced numbers and/or dysfunction of circulating EPCs, while an expanded EPC pool is associated with a decreased cardiovascular mortality. (1, 7, 78, 264, 265). EPC numbers in patients with T2DM have been shown to be significantly lower than in non-diabetic subjects(86) and the levels of EPC acts as a surrogate biological marker for vascular function and cumulative cardiovascular risk(7). ROS in diabetes plays an important role in down-regulating hypoxia inducible factor (HIF)-1 α resulting in inability to mobilize EPC from the bone marrow in response to tissue ischaemia(172, 174, 175).

There are only limited data on the effect of taurine supplementation and EPC. Taurine has been reported to suppress sympathetic tone and attenuates EPC senescence(270). Fennessy et al evaluated the monocyte-endothelial interactions using the monocyte derived from young smokers (monocyte-condition medium, MDM) on human umbilical vein endothelial cells (HUVECs). In that study, taurine up-regulated the level of nitric oxide synthase expression, normalized the level of NO, suppressed endothelin-1 and contributes to improvement in flow-mediated dilatation (FMD) of brachial artery in young smokers(263). In that study, monocytes were not cultured for the development of EPC, hence the numbers and functions of EPC were not assessed.

The first study of taurine intervention in diabetes was published by Moloney et al who demonstrated that 2 weeks of taurine supplementation in Type 1 diabetes patients, improved flow mediated dilatation (FMD), pulse wave velocity (PWV) and augmentation index (AIx)(16), a well-validated, non-invasive technique to evaluate endothelial dysfunction and arterial stiffness(196, 271).

We hypothesized that taurine supplementation may have similar benefits in T2DM patients. We carried out a pilot study using a randomized, double blind, cross-over placebo design. We examined the effects on endothelial dysfunction by measuring the levels of EPCs and PWV.

3.2 Methods

Participants were identified when they attended routine clinic visits at the diabetes outpatient clinic and the diabetes day centre at Connolly Hospital from December 2012 to April 2013. The study was discussed and a patient information leaflet was given (Appendix section 4.2.1). Participants who agreed to the study were given an appointment to attend the diabetes day centre, where full informed consent was obtained.

The inclusion criteria included stable, well controlled type 2 diabetes ($HbA1c < 6.5\%$ or 48mmol/mol), on diet treatment alone or on stable dose of anti-diabetic treatment/s for the last 3 months, and aged between 18 to ≤ 65 years old.

Participants with evidence of renal impairment ($cc < 60\text{ mL/min}$), presence of microalbuminuria, having intercurrent illness, uncontrolled hypertension (treated or untreated), foot ulcer, any retinopathy, active infection, any malignancy, recent (within last 6 months) myocardial infarction, heart failure, or stroke, peripheral vascular disease or absent peripheral pulses, history of psoriasis, seizure or epilepsy, previous hypersensitivity to taurine supplement, pregnant and breast feeding women, children or minors under 18 years old, and cognitively impaired persons were excluded from the study.

All participants signed an informed consent (Appendix section 4.2.2) and this study was approved by the Connolly Hospital Ethics Committee (Appendix section 4.2.3)

On the first visit (day 0), participants had anthropological measurements such as weight, height, body mass index (BMI), waist circumference, hip circumference, waist to hip ratio (WHR), clinical assessment (medical history, medications, blood pressure), routine fasting blood testing (renal/ liver/ bone profile, HbA_{1c}, cholesterol), blood sampling for EPC culture, and carotid femoral PWV. Height and weight were measured without shoes and rounded to the closest 0.5cm and nearest kg. Waist circumference was measured using non-stretchable tape, while standing, feet together, midpoint between lower rib margin and iliac crest.

By using a random number generator, each participant was randomly assigned either Taurine 500mg or placebo supplementation three times daily. Participants were advised not to change any dietary habits during the study.

On the intermediate visit (day 30), participants had similar measurements, clinical assessments, blood tests including extra sample for EPC evaluation and PWV measured. Any remaining tablets were counted as a proxy to evaluate compliance. After 2 weeks washout, participants were then assigned in a crossover fashion to the opposite supplement for another 30 days.

These tests were repeated when participants returned for the final visit (day 60). Throughout this time, participants were free to contact the investigators if they become unwell after taking either supplement.

A person independent of the study was assigned to manage the randomization code, storage, dispensing and recording supplements assigned to participants at baseline and intermediate visit. This code was kept securely and the participant was only unblinded in the event of an adverse event.

At each visit, participants had blood drawn for endothelial progenitor cells analysis (EPC). The method for EPC cells culture and adhesion assay was described previously (Section 4.3).

A single operator measured the Carotid-Femoral PWV (cfPWV) in all participants. This was performed under a standardized condition as described by the 2006 expert consensus(198). Participants fasted from midnight and refrained from smoking 3 hours before measurement. The test was performed in a supine position, after 10 minutes rest, in a quiet room with stable temperature of 21⁰c. We used the Vicorder® Vascular Complete device (Skidmore Medical, Bristol UK) which uses oscillometric techniques to detect a pulse waveform. This device has been validated against the SphygmoCor device in various studies(272-275). To detect the femoral pulse, a 100mm wide blood pressure cuff was placed around the right upper thigh while a smaller 30mm partial cuff was placed around the neck at the level of right carotid artery for the detection of carotid pulse. Blood pressure and the distance between suprasternal notch to the upper limit of femoral cuff were measured and recorded in the software provided. Each participant had three measurements performed and average readings were calculated and used for analysis. PWV is expressed in meter per seconds (m/s).

Taurine capsules were sourced from Lambert Healthcare Ltd (UK), manufactured by Thompson and Capper Ltd (Appendix section 4.2.4) while placebo (Microcrystalline Cellulose) capsules were sourced from Mawdsley Brooks and Co (UK) (Appendix 4.2.4). Taurine was encapsulated in Hydroxypropyl Methylcellulose (HPMC) while placebo was encapsulated in gelatin. Both are common material used for capsule manufacturing and are known to mask taste and odour from active ingredient(276, 277). Only the independently assigned person responsible for managing the randomization code had direct access to both Taurine and placebo capsules. Neither company provided any financial support to this study.

3.3 Statistical Analysis

Data were analyzed based on treatment group. Normally distributed data are expressed in mean \pm SD while skewed data are expressed in median and interquartile range (IQ range). One-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer was used when comparing normally distributed data between groups while Kruskal-Wallis test was used for skewed data. The $p < 0.05$ was set as a significant level. All participants were entered into the study on an intention-to-treat basis and no subject was removed or dropped from the study.

3.4 Results

A total of 11 participants, 8 males and 3 females, were enrolled in the study. Due to technical reasons, 1 participant did not have the EPC result after Taurine supplementation while 1 participant did not have EPC adhesion study at baseline.

Table 3-1 demonstrates the baseline characteristics of all participants in the study. The median age was 59.7 (56.7 - 61.7) years old with a mean diabetes duration was 4.4 ± 3 years. Mean waist-hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), LDL cholesterol (LDL) and triglycerides (TG) were 0.96 ± 0.06 , 138.7 ± 18.2 mmHg, 86.3 ± 10.2 mmHg, 1.97 ± 0.63 mmol/L and 1.81 ± 1.12 mmol/L. Median body mass index (BMI), glycated Haemoglobin (HbA_{1c}), total cholesterol (TC), and HDL cholesterol (HDL), were 30.5 ($27.8 - 32.7$) kg/m², 44 ($43-48$) mmol/mol, 4.2 ($3.5-4.9$) mmol/L, and 1.4 ($1.31-1.5$) mmol/L. Majority of the participants (81.8%) were on aspirin, statin and metformin while only 4 (36.4%) participants were on antihypertensive/s including Angiotensin Converting Enzyme Inhibitor (ACEI) or Angiotensin Receptor Blocker (ARB). A total of 2 participants (18.2%) were smoker at baseline. Baseline PWV was 8.7 ($8.0-10.7$) m/s while total EPC per high power field (EPC/hpf) and EPC adhesion capacity were 32 ± 8 cells/hpf and 0.66 ± 0.4 fluorescence unit respectively.

Table 3-1: Parameters at baseline. Normally distributed data are expressed as mean \pm SD while skewed data are expressed as median (interquartile range, IQ range). ACEI (Angiotensin Converting Enzyme Inhibitor), ARB (Angiotensin Receptor Blocker), BMI (Body Mass Index), HbA_{1c} (Glycated Haemoglobin), ACR (Albumin Creatinine Ratio), HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), EPC (Endothelial Progenitor Cells).

Baseline Characteristics	Values
Total participants	11
Age (years)	59.7 (56.7-61.7)
Diabetes Duration (n, years)	9 (4.4 \pm 3.0)
Gender (n, %)	
Male	8 (72.7)
Female	3 (27.3)
BMI (kg/m ²)	30.5 (27.8 – 32.7)
Waist Hip Ratio (WHR)	0.96 \pm 0.06
Systolic Blood Pressure (mmHg)	138.7 \pm 18.2
Diastolic Blood Pressure (mmHg)	86.3 \pm 10.2
Concomitant Medications (n, %)	
Statin	9 (81.8)
Aspirin	9 (81.8)
Antihypertensive	4 (36.4)
ACEi or ARB	4 (36.4)
Metformin	9 (81.8)
Smoker (n, %)	2 (18.2)
Fasting glucose (mmol/L)	6.33 \pm 0.97
Glycated Haemoglobin, HbA _{1c} (mmol/mol)	44 (43-48)
Creatinine (mmol/L)	70.3 \pm 9.3
Urine ACR (mg/mmol)	1.02 \pm 0.38

Baseline Characteristics	Values
Total Cholesterol (mmol/L)	4.2 (3.5-4.9)
HDL Cholesterol (mmol/L)	1.4 (1.3-1.5)
LDL Cholesterol (n, mmol/L)	10, 2.0 \pm 0.6
Triglycerides (mmol/L)	1.8 \pm 1.1
Pulse Wave Velocity (m/s)	8.7 (8.0-8.4)
EPC per high power field (EPC/hpf, cells)	32 \pm 8
EPC adhesion (fluorescence unit)	10, 0.66 \pm 0.4

Table 3-2 demonstrates the comparison of measured parameters between baseline, post taurine and post placebo. There were no significant differences in body mass index (BMI), waist-hip ratio (WHR), SBP, DBP, Creatinine, Alb/Creat ratio, fasting glucose, HbA_{1c}, TC, HDL, LDL, TG levels between groups.

There was a trend towards improvement in the number of EPC/hpf following taurine (40 \pm 7 cells) and placebo (37 \pm 10 cells) when compared to baseline (32 \pm 8 cells, $p=0.08$) (Table 3-2, Figure 3-1). Similarly, there was a trend towards improvement in DBP following taurine (78.6 \pm 7.4 mmHg) and placebo (79.3 \pm 7.5 mmHg) when compared to baseline (86.3 \pm 10.2mmHg, $p=0.08$) (Table 3-2, Figure 3-2). There were no differences in the EPC adhesion capacity between baseline ($n=10$, 0.66 \pm 0.4 fluorescence unit), taurine (10, 0.97 \pm 0.8 fluorescence unit) and placebo (11, 0.94 \pm 0.5, $p=0.43$ fluorescence unit) group. There were also no differences in the measurements of PWV baseline [8.7 (8.0-8.4) m/s], taurine [9.1 (8.3-9.6) m/s] and placebo [8.9 (8.8-9.1) m/s, $p=1.0$] (Table 3-2).

Table 3-2 Comparison between changes in parameters at baseline, after Taurine and Placebo supplementations. Normal data are expressed as mean \pm SD while skewed data are expressed as median (interquartile range, IQ range). Unless stated (n) before mean and median values, there are 11 participants per group. Trends of improvement in DBP and EPC numbers per hpf following intervention. BMI (body mas index), WHR (waist-hip ratio), SBP (systolic blood pressure), DBP (diastolic blood pressure), Creat (Creatinine), Alb (Albumin), HbA_{1c} (Glycated Haemoglobin), TC (Total Cholesterol), HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), TG (Triglycerides), PVW (Pulse Wave Velocity), EPC (Endothelial Progenitor Cell), hpf (high power field). Significant difference at p value < 0.05.

Parameters	Baseline	Taurine	Placebo	Sig
Age (years)	59.7 (56.7-61.7)	-	-	-
Compliance (%)	-	95.2 (92.8-100)	96.8 (86.5-100)	0.72
BMI (kg/m ²)	35.2 \pm 5.1	34.9 \pm 4.8	35.6 \pm 5.7	0.96
WHR	0.96 \pm 0.06	0.97 \pm 0.06	0.96 \pm 0.05	0.87
SBP (mmHg)	138.7 \pm 18.2	132.1 \pm 15.2	136 \pm 17	0.65
DBP (mmHg)	86.3 \pm 10.2	78.6 \pm 7.4	79.3 \pm 7.5	0.08
Creat (μ mol/L)	70.3 \pm 9.3	67.7 \pm 10.7	69.6 \pm 11	0.84
Alb/Creat ratio (n, mg/mmol)	11, 1.02 \pm 0.38	8, 1.01 \pm 0.51	10, 1.09 \pm 0.45	0.91
Fasting glucose (n, mmol/L)	11, 6.33 \pm 0.97	8, 6.23 \pm 0.62	9, 61.9 \pm 0.9	0.93
HbA _{1c} (mmol/mol)	44 (43-48)	44 (43-47)	44 (43-47)	0.99
TC (mmol/L)	4.2 (3.5-4.9)	3.8 (3.6-4.7)	3.9 (3.4-4.5)	0.65
HDL (mmol/L)	1.4 (1.3-1.5)	1.4 (1.2-1.6)	1.4 (1.2-1.4)	0.97
LDL (n, mmol/L)	10, 2.0 \pm 0.6	11, 2.1 \pm 0.7	11, 2.1 \pm 0.8	0.88
TG (mmol/L)	1.8 \pm 1.1	1.6 \pm 0.7	1.4 \pm 0.8	0.5
PWV (m/s)	8.7 (8.0-8.4)	9.1 (8.3-9.6)	8.9 (8.8-9.1)	1.0

Parameters	Baseline	Taurine	Placebo	Sig
EPC/hpf (n, cells)	11, 32 ± 8	10, 40 ± 7	11, 37 ± 10	0.08
EPC Adhesion (n, fluorescence unit)	10, 0.66 ± 0.4	10, 0.97 ± 0.8	11, 0.94 ± 0.5	0.43

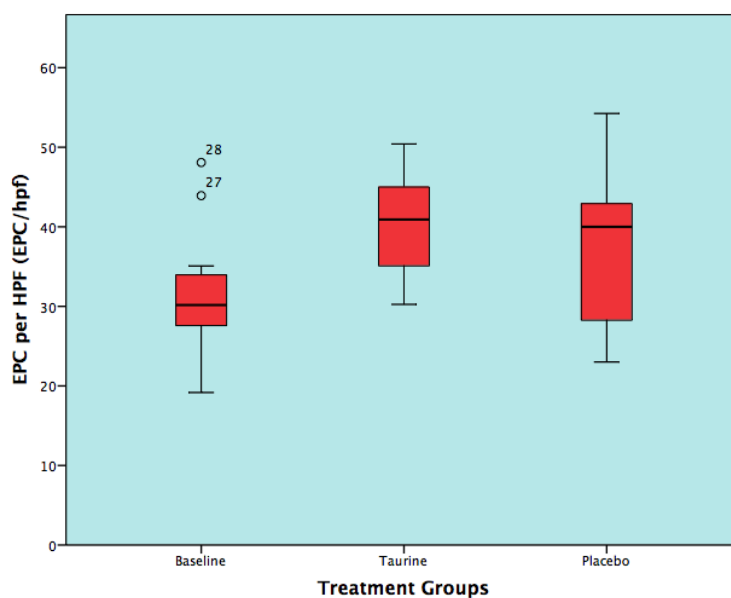


Figure 3-1 Comparing numbers of EPC per high power field (EPC/hpf) based on treatment groups (Study 2). Single horizontal line in the bar represents mean value while error bar represent 95% confidence interval. Clear circles represent outliers. There was a trend of improvement in the number of Endothelial Progenitor Cells (EPC) per high power fields (hpf) following intervention with $p = 0.08$.

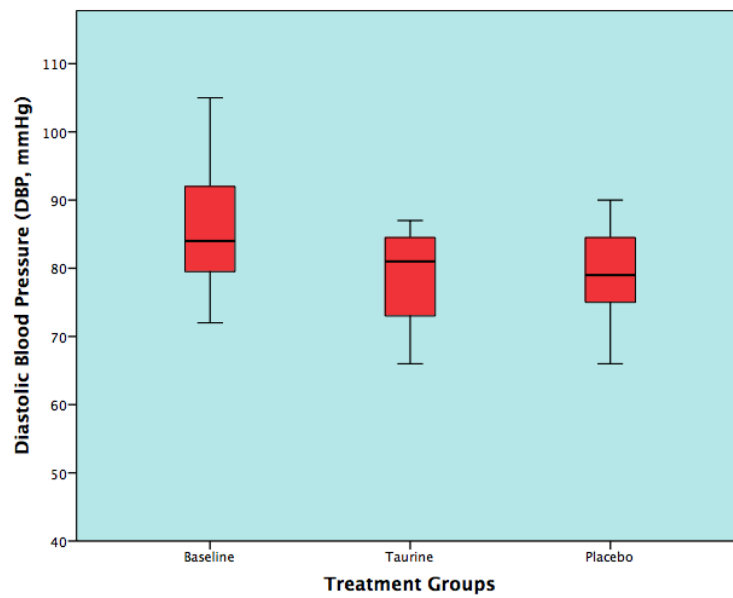


Figure 3-2 Comparing diastolic blood pressure (DBP) based on treatment groups (Study 2). Single horizontal line in the bar represents mean value while error bar represent 95% confidence interval. There was a trend of improvement in Diastolic Blood Pressure following intervention with $p=0.08$.

3.5 Discussion

In this pilot study, we demonstrated no significant difference between the effects of Taurine and placebo supplementation on markers of endothelial dysfunction and arterial stiffness assessed using EPC and PWV in patients with T2DM. This result may be interpreted as an absence of benefit of Taurine in modulating known markers of endothelial dysfunction in T2DM. However, we also demonstrated a downward trend in DBP and an upward trend in the numbers of EPC with intervention.

To our knowledge, this is the first study evaluating the effect of Taurine on EPC and arterial stiffness in patients with T2DM. Two previous taurine intervention studies in T2DM evaluated the effects of taurine on glycaemic control and biomarkers of fibrosis(258, 278).

3.5.1 Comparison with T1DM Study

The closest study to compare to, involved participants with T1DM as published by Moloney et al. (16).

Our cohort of T2DM was older but had better overall glycaemic control. Our cohort had higher BMI while participants with BMI $\geq 30\text{kg/m}^2$ were excluded from the T1DM study. Similarly, 2 of our participants were smokers and continued to smoke throughout the study while smoker was excluded in the T1DM study. Concomitant medications were not reported in the T1DM study, while most of our participants were on statin, aspirin, metformin and antihypertensive at baseline.

These unavoidable differences may contribute to the differences in the degree of arterial stiffness in our cohort compared to the T1DM cohort. Our cohort is likely to have longer duration of arterial stiffness as demonstrated by studies showing a stepwise progression from pre-diabetes state towards diabetes and the strong association between arterial stiffness and insulin resistance(279-281).

For the measurement of arterial stiffness, instead of Augmentation Index (AIx) and Flow-Mediated Dilatation (FMD), we performed PWV, as it is

the current gold standard, especially in subjects with diabetes(197). Additionally, our device was less-operator dependent, well validated, and have been shown to be highly reproducible (272-275, 282).

We used a similar dose of taurine, allowed a similar washout period and performed similar comparison analyses between the groups of treatment. As each participant acts as his/her own control, we elected not to recruit a control, non-diabetic cohort.

Similar to other studies(16, 242), we did not perform a second baseline after two weeks washout, as it was considered adequate for complete elimination of active treatment(241, 242).

3.5.2 Discussion of Literature in Relation to Study Results

Our result did not show any significant difference to the markers of endothelial dysfunction (EPC and PWV) following supplementation with taurine compared to placebo and baseline.

Although there is an abundance of evidence linking diabetes and arterial stiffness, there have been only a few studies examining the changes in arterial stiffness with intervention in T2DM subjects(197). A systematic review of trials with Pioglitazone demonstrated a composite benefit in arterial stiffness as measured using PWV(283). More recently, Davenport et al demonstrated that statin improves arterial stiffness, measured using PWV at 3 and 12 months follow up with no dose-dependent effect observed(284). The results from our intervention study in T2DM will add to the body of evidence in this area.

The trend of improvement in EPC and DBP following intervention especially with taurine was intriguing. Previous animal studies did demonstrate that taurine depletion leads to salt-induced hypertension while supplementation of taurine attenuates this response(237, 285, 286). However, there are very limited clinical studies on the antihypertensive effect of taurine on blood pressure(10). One such study demonstrated a reduction of SBP and DBP by 9mmHg and 4mmHg respectively after 6g/day of taurine supplementation for 1 week(287). In a recent

prospective, double-blinded, placebo control study, 120 pre-hypertensive, non-diabetic participants were randomized to either taurine 1.6g/day for 12 weeks demonstrated an improvement in the clinic and 24 hour ambulatory blood pressure measurement, compared to placebo(11). In the same study, taurine also improves the level a vasodilator hydrogen sulfide (H_2S), another potential mechanism of the antihypertensive effect of taurine (11). Furthermore, in an animal model of diabetes, restoration of H_2S in improves wound healing in by restoring EPC function(288). Further clinical studies involving intervention with taurine in T2DM with measurements of H_2S would be the next important step to characterize the potential benefit of taurine in this group.

The optimum dose for taurine supplementation in order to exert any clinical effect is not known. Taurine doses up to 3g per day have been shown to elevate plasma taurine level in T2DM without any effect on glycaemic control(258). It is not known if the same dose or more is needed to have any significant effect on EPC or arterial stiffness in patients with T2DM. The dose of 1.5g per day is the most commonly used in other human studies (16, 242, 260, 263), although it is a lower dose when compared to animal studies where taurine dose may reach more than 10 times per body weight of the animal(9).

Despite previous experimental data supporting its role as an antioxidant, the effect of taurine on EPC has never been studied. Although Fennessy et al used similar techniques to our study, in a group of young smokers(263), the cells were not cultured to the level of EPC, so comparisons with our study cannot be made.

While EPC and PWV are independent early predictors of CVD, in a lower risk group, EPC continues to predict CVD better than the Framingham score (6, 7, 38, 78). However, only several studies are available where EPCs were measured with PWV in patients with diabetes(222-224). Similarly, only limited publications are available, where improvement of PWV with intervention were demonstrated in T2DM, mostly derived from a high-risk diabetes subjects(197, 283, 284). For this pilot study, our

participants were of T2DM patients with low-risk for CVD. At baseline, our participants had excellent blood pressure control, no microalbuminuria and a median cfPWV of 8.7 (IQ 8.0-8.4) m/s which was lower than the 2010 (202) and the 2011 recommended threshold of 10m/s (201). The majority of our participants were on multidrug therapy including statins, which have been associated with 15% to 35% improvement of EPC(82-85, 289). It is possible that in this low risk group, with multidrug treatment, the EPC pool is recovering. Although there was a trend of EPC improvement with intervention, there was no difference in PWV suggestive of no changes in arterial stiffness, at least in our cohort. Our data adds to the limited publications available examining the link between EPC and PWV with intervention.

3.5.3 Limitations and Strengths

Our sample size was small and our study duration in each intervention was short. We could not outrule a potential type-1 error contributing to the downward trend in DBP and an upward trend in the numbers of EPC with intervention. As this was a pilot study in T2DM, we based it on the previously published T1DM study by Moloney et al, who had fewer participants. Our study will serve as a preliminary to future studies in Taurine with T2DM.

We did not perform a second baseline measurement after the 2 weeks washout period and although previous publications support the duration of active drug withdrawal as being adequate, we cannot completely outrule the possibility of remaining active ingredient affecting our results. We strongly advised our participants not to change their dietary habit during the study. However, by not measuring serum taurine, we cannot outrule any influence if any, of dietary taurine on the results. Future studies needs to involve more participants preferably with higher risk T2DM, in a prospective placebo control design and with the measurement of serum and urinary excretion of taurine.

Our study is the first to evaluate the effects of taurine on EPC and arterial stiffness in T2DM participants. There is insufficient evidence

characterizing the interaction between reversibility of arterial stiffness with intervention, the effects of taurine on EPC and blood pressure, and the interaction between EPC and PWV. The trend of improvement of EPC and DBP following intervention, especially with taurine, potentially through the restoration of H₂S needs to be further examined.

3.5.4 Summary

In summary, in comparison to placebo, supplementation of taurine for 30 days did not exert any significant effects on EPC numbers, adhesion capacity, and PWV, which are known markers of endothelial dysfunction. More studies are needed with bigger sample size, longer duration, and with different risk factor profiles to further characterize any potential role of taurine as a modulator of CV risk in T2DM.

4 Appendix

4.1 Study 1

4.1.1 Patient Information Leaflet



Patient Information Leaflet:

The Effect of DPP4 inhibitor or Sulphonylurea on Endothelial Progenitor Cells in Type 2 Diabetic Patients

Cardiovascular disease (e.g heart attack, or stroke) are important complications of diabetes. Controlling risk factors for these complications, such as high cholesterol and high blood pressure, and stopping smoking have been shown to reduce the risk of developing these complications.

Endothelial Progenitor Cells (EPCs) are cells which circulate in the blood which are thought to be responsible for forming new blood vessels in adults if necessary and also repairing any damage to blood vessels. There is limited information regarding the effect of different diabetes treatments on the number of EPCs in the bloodstream.

Sulphonylurea (Gliclazide MR) is a well-established anti-diabetic medications used for the last 60 years. It is licensed as a second line tablet for treatment of diabetes.

DPP4 inhibitor (Saxagliptin) is a new medication for diabetes patients. It is a tablet that has been proven to be very effective in reducing blood

sugar level. It is licensed to be use as a second line medication in treating diabetes.

We plan to examine the effect of Saxagliptin and Gliclazide MR on EPC number and function in patients with diabetes. The results of the study may give us a clue as to the effects of these medications on the risk of having cardiovascular disease in the future.

You have been invited to participate in this study as you need additional treatment for your diabetes, and either Gliclazide or Saxagliptin have been considered to be good treatment choices for you. If you agree to take part in this study, you will be allocated randomly to either receive Gliclazide MR or Saxagliptin.

The most common side effects from Gliclazide MR are low blood sugar (hypoglycaemia) and gastrointestinal disturbance. You will need to check your blood sugar more frequently while on this medication. Saxagliptin may also cause low blood sugar but not as frequently as Gliclazide MR. It may also cause gastrointestinal disturbance, dizziness and muscle aches. Both medications are well tolerated in general.

Before commencing treatment, we will perform routine clinical examination including examination of your eyes, recording blood pressure, height, weight and waist to hip ratio. We will take blood samples to grow EPCs and we will also check cholesterol, glucose, HbA1c, kidney, liver, and bone profile level. After 6 months, you will have similar clinical examinations and similar blood sample will be taken. All of this should not take much more than 30 minutes at each visit. All of your details will be kept confidential. There will be no need for any further visits apart from your usual hospital visits.

Participation in this study is entirely voluntary and you may withdraw at any time, without giving reason, and without this decision effecting any future treatment or medical care.

Our responsibilities as investigators:

If you experience any ill effects as the result of your participation, then we will take care of your medical needs. If we discover any medical problems such as high blood pressure or cholesterol, this would be discussed with you in detail, and you would be offered appropriate treatment and follow up in a specialist clinic or with your GP.

If there are any new findings during the course of the study that may affect the validity of the study or your participation in it, then the findings will be made available to you and you will receive the appropriate medical care and advice depending on the test results.

Confidentiality:

Only anonymous data, ie. data that do not identify you by name, will be collected. Only authorised representatives of the study team will have direct access to your personal medical records. All study team representatives and medical staff are obliged to maintain confidentiality at all times. The data shall be destroyed by shredding and deletion of computer files at the end of the study

For additional information now or any future time please contact:

Dr. Wan Aizad Wan Mahmood, Research Registrar

Connolly Hospital, Blanchardstown, Dublin 15. Phone: 01 646 5756

Dr. John McDermott, Consultant Endocrinologist

Connolly Hospital, Blanchardstown, Dublin 15. Phone: 01 646 5758

4.1.2 Consent Form



CONSENT FORM

Protocol Title: The Effect of DPP4 inhibitor or Sulphonylurea on Endothelial Progenitor Cells in Type 2 Diabetic Patients

Please tick the appropriate answer.

- I consent to take part in this study. ☐ **Yes** ☐ **No**

- I confirm that I have read and understood the Patient Information Leaflet attached (version 1, 23/08/2010), and that I have had ample opportunity to ask questions all of which have been satisfactorily answered.

☐ **Yes** ☐ **No**

- I have been given a copy of the Patient Information Leaflet and this Consent form for my records.

☐ **Yes** ☐ **No**

- I understand that my participation in this study is entirely **voluntary** and that I may withdraw at any time, without giving reason, and

without this decision affecting my future treatment or medical care.

☐ **Yes** ☐ **No**

- I understand that my medical records may be viewed by members of the research team

☐ **Yes** ☐ **No**

- I understand that my identity will remain confidential at all times.

☐ **Yes** ☐ **No**

- I am aware of the potential risks of this study.

☐ **Yes** ☐ **No**

- I agree that I will not restrict the use to which the results of this study may be put. I give my approval that anonymous data concerning my person may be stored or electronically processed for the purpose of scientific research and may be used in related or other studies in the future. (This would be subject to approval by an independent body which safeguards the welfare and rights of people in biomedical research studies - the Connolly Hospital Ethics (Medical Research) Committee.)

☐ **Yes** ☐ **No**

- I consent for further follow-up studies

☐ **Yes** ☐ **No**

Patient _____

Signature and dated

Name in block capitals

To be completed by the Principal Investigator or his nominee.

I the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, as well as the possible benefits and have invited him/her to ask questions on any aspect of the study.

Signature, qualifications and date

Name in block capitals

4.1.3 Poster publication



Cardiovascular Safety of DPP-4 inhibition in Patients with Type 2 Diabetes Mellitus: Endothelial Progenitor Cells as an Early Marker of Long-term Cardiovascular risk

WA Wan Mahmood, TF King, T Kyaw Tun, S Sreenan, JH McDermott
Department of Endocrinology and Diabetes, Connolly Hospital, Blanchardstown,
Dublin 15, Ireland

Disclosure: Authors have no potential conflict of interest



BACKGROUND

Endothelial Progenitor Cells (EPCs) are circulating bone-marrow derived cells which promote post-natal vasculogenesis.

EPCs play a central role in cardiovascular repair and studies have demonstrated a link between EPC number and function and future cardiovascular risk [1].

Interventions which modify cardiovascular risk have early effects on EPC number and function [2].

Recently there has been increased appreciation of the need to evaluate cardiovascular safety of new anti-diabetic agents, but long-term post-marketing trials are often required before an adverse cardiovascular signal is seen.

DPP-4 inhibitors are effective glucose-lowering agents, but their long-term cardiovascular safety has not been extensively tested.

We aimed to evaluate the long-term cardiovascular safety of Saxagliptin (SAX, a DPP-4 inhibitor) versus Gliclazide Modified Release (GLC) using EPC number and function as surrogate markers of cardiovascular risk

PATIENTS AND METHODS

18 patients with T2DM on Metformin monotherapy requiring diabetes treatment intensification were randomized to SAX (n=7) or GLC (n=11) in an open-label fashion.

EPC number and adhesion capacity were measured, using methods as previously described [3], at baseline and 6 months after treatment.

At each visit, additional fasting blood tests for HbA1c, renal profile, glucose, fasting lipids and urine albumin creatinine ratio (UAC) were performed.

Table 1: Comparing baseline parameters based on treatment. Table 2: Comparing treatment group at 6 months to baseline
BMI = Body Mass Index, WHR = waist-hip ratio, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, HbA_{1c} = Glycosylated Haemoglobin.
Significance value p<0.05.

RESULTS

Table 1	Saxagliptin (n = 7)	Gliclazide MR (n = 11)	P value
Gender, n(%)			
Female	1 (14.3)	5 (45.5)	0.39
Male	6 (85.7)	6 (54.5)	
Smoker, n(%)	0	3 (30)	0.34
Age (mean ± SD years)	57.89 ± 6.1	55.53 ± 8.61	0.54
BMI (mean ± SD kg/m ²)	29.35 ± 1.77	30.67 ± 3.37	0.36
WHR (mean ± SD)	0.94 ± 0.33	0.97 ± 0.83	0.33
SBP (mean ± SD mmHg)	145.14 ± 28.1	139.45 ± 19.74	0.62
DBP (mean ± SD mmHg)	88.43 ± 14.52	83.91 ± 10.68	0.46
Diabetes duration (mean ± SD years)	4.86 ± 2.67	3.64 ± 2.01	0.29
Creatinine (mean ± SD years) mmol/L	88.9 ± 15.2	63.5 ± 14.5	0.003
Glucose (mean ± SD years) mmol/L	7.54 ± 0.73	8.81 ± 1.75	0.16
HbA _{1c} (mean ± SD years) mmol/mol	53.1 ± 4.4	62.2 ± 8.8	0.02
Total EPC per HPF [med (IQ range)] cells	35 (21 – 54)	25 (22 – 46)	0.6
EPC Adhesion capacity [med (IQ range)] fluorescence units	0.22 (0.17 – 0.85)	0.27 (0.1 – 0.67)	0.8

Table 2	Saxagliptin (n = 7)	Gliclazide MR (n = 11)
HbA1c (mean ± SD), mmol/mol		
Baseline	53.1 ± 4.4	62.9 ± 9.0
6 months	50.4 ± 6.6	58 ± 8.3
Sig	0.33	0.13
Weight (mean ± SD), kg		
Baseline	84.2 ± 10	86 ± 14.7
6 months	84.5 ± 9.6	87.9 ± 14.9
Sig	0.8	0.008
EPC number [med (IQ range)], cells/HPF		
Baseline	35 (21 – 54)	25 (22 – 46)
6 months	35 (28 – 38)	39 (28 – 46)
Sig	0.5	0.24
EPC adhesion capacity [med (IQ range)], fluorescence units		
Baseline	0.2 (0.17 – 0.86)	0.27 (0.1 – 0.67)
6 months	0.3 (0.17 – 0.65)	0.32 (0.14 – 0.84)
Sig	1.0	0.9

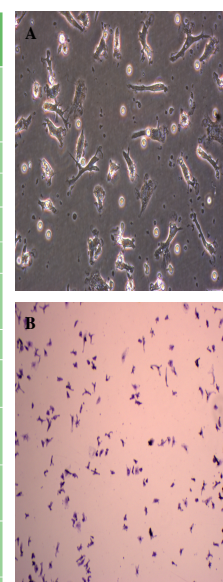


Figure 1A : EPC at high power field (400x).
Figure 1B : EPC adhesion assay (100x)

SUMMARY

We found no difference in EPC numbers or adhesion capacity in patients treated with Saxagliptin vs Gliclazide MR for 6 months

This may suggest a similar cardiovascular safety profile of Saxagliptin to Gliclazide MR, a well-established treatment for type 2 diabetes

REFERENCES

1. Vasa, M., et al., Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001; **89**(1): p. E1-7.
2. Liew, A., F. Barry, and T. O'Brien. Endothelial progenitor cells: diagnostic and therapeutic considerations. *Bioassays*. 2006; **28**(3): p. 261-70.
3. King, T.F., et al., Endothelial progenitor cells in mothers of low-birthweight infants: a link between defective placental vascularization and increased cardiovascular risk? *J Clin Endocrinol Metab*. 2013; **96**(1): p. E33-9.

4.2 Study 2

4.2.1 Patient Information Leaflet



Patient Information Leaflet:

The Effect of Taurine on Endothelial Dysfunction in Patients with Type 2 Diabetes.

Diabetes is a chronic condition associated with complications such as blindness, kidney failure, impaired sensation, heart attack and strokes. The risk of having stroke and heart attack in type 2 diabetes (T2DM) patients is increased by two to three fold compared to people without diabetes.

Central to this complication is the blood vessel. Cells called endothelial cells form blood vessels. Blood vessel problems (i.e. stiff or narrowed blood vessel) are more common in diabetes patients and contribute to heart attack and strokes.

Taurine is a type of protein present in seafood and has been shown in some studies to be protective for endothelial cells against damage. Endothelial Progenitor Cells (EPCs) are cells that come from bone marrow and mature into endothelial cells. They circulate in the blood and they are thought to be responsible for forming new blood vessels in adults and also repairing any damage to blood vessels. Patients with diabetes have lower EPCs number compared to non-diabetic patients, which may lead to blood vessel dysfunction subsequently heart attack and strokes.

Recently, a group of researcher from Royal College of Surgeons in Ireland (RCSI) have shown that supplementation of taurine three times daily improves the function of blood vessel and improve the function of EPCs in Type 1 diabetes patients.

We plan to replicate a similar study in Type 2 diabetes patients. We want to see if taurine supplementation in Type 2 diabetes patients will show the same effects.

You have been invited to participate in this study, as you appear to be a suitable patient based on predefined criteria.

This study is a double blinded randomized cross-over design. This means that you will be given either taurine capsule or placebo capsule for 30 days followed by placebo capsule or taurine capsule for another 30 days. There will be a 2-week period of neither capsule in between. All capsules will be provided.

As this is a double-blind study, neither you nor the investigators will know which supplement you are on at any time. At the beginning, at 30 and 60 days of the study, you will have your usual tests (i.e weight, height, blood pressure etc.), blood tests to measure markers of blood vessel health and extra bloods for EPCs study. You will undergo a 5-minute assessment of how stiff your blood vessel by wearing a blood pressure cuff on your thigh and on your neck. You will continue taking your other medications as usual throughout the study.

There is a very low risk of complications with blood test. By adhering to the hospital policy on blood letting, the risk of infection is very small. Minor bruising may be seen and this should resolve within a few days. Our researcher is very experience in taking blood, so the risk is very small. The blood pressure cuff on the thigh and neck will inflate during the measurement of your artery. The pressure is small and will not cause any discomfort to you.

Taurine is a naturally available protein from seafood. Similar doses of taurine have been used in clinical trials for up to a year without any adverse effect. Patients with kidney failure, seizure disorder, psoriasis skin disease may have the potential to develop side effect from taurine namely skin itching, nausea, headache and dizziness. The fact that you are invited to participate in the study means that we have excluded you from those conditions and your likelihood of having the side effects therefore is very low.

Participation in this study is entirely voluntary and you may withdraw at any time, without giving reason, and without this decision effecting any future treatment or medical care.

Our responsibilities as investigators:

If you experience any ill effects as the result of your participation, then we will take care of your medical needs. Your treatment will be unblinded so we can verify if these ill effects are due to the study medication.

If we discover any medical problems such as high blood pressure or cholesterol, they will be discussed with you in detail, and you will be offered appropriate treatment and follow up in a specialist clinic or with your GP.

If there are any new findings during the course of the study that may affect the validity of the study or your participation in it, then the findings will be made available to you and you will receive the appropriate medical care and advice depending on the test results.

Confidentiality:

Only anonymous data, ie. data that do not identify you by name will be collected. Only authorised representatives of the study team will have direct access to your personal medical records. All study team representatives and medical staffs are obliged to maintain confidentiality at all times. Although we hope to publish the results at the end of the

study, no information that is traceable to you will be included in any such publication.

For additional information now or any future time please contact:

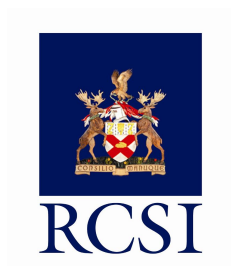
Dr. Wan Aizad Wan Mahmood, Research Registrar

Connolly Hospital, Blanchardstown, Dublin 15. Phone: 086-0229898

Dr. Colin Davenport, Research Specialist Registrar (SpR)

Beaumont Hospital, Dublin 9. Phone: 01-8092377

4.2.2 Consent form



CONSENT FORM

Protocol Title: The Effect of Taurine on Endothelial Dysfunction in Type 2 Diabetic Patients

Please tick the appropriate answer.

- I consent to take part in this study. ☐ **Yes** ☐ **No**
- I confirm that I have read and understood the Patient Information Leaflet attached (version 2, 18/11/2011), and that I have had ample opportunity to ask questions all of which have been satisfactorily answered. ☐ **Yes** ☐ **No**
- I have been given a copy of the Patient Information Leaflet and this Consent form for my records. ☐ **Yes** ☐ **No**
- I understand that my participation in this study is entirely **voluntary** and that I may withdraw at any time, without giving reason, and

without this decision affecting my future treatment or medical care.

☐ **Yes** ☐ **No**

- I understand that my medical records may be viewed by members of the research team

☐ **Yes** ☐ **No**

- I understand that my identity will remain confidential at all times.

☐ **Yes** ☐ **No**

- I am aware of the potential risks of this study.

☐ **Yes** ☐ **No**

- I agree that I will not restrict the use to which the results of this study may be put. I give my approval that anonymous data concerning my person may be stored or electronically processed for the purpose of scientific research and may be used in related or other studies in the future. (This would be subject to approval by an independent body which safeguards the welfare and rights of people in biomedical research studies - the Connolly Hospital and Beaumont Hospital Ethics (Medical Research) Committee.)

☐ **Yes** ☐ **No**

Patient _____

Signature and dated

Name in block capitals


To be completed by the Principal Investigator or his nominee.

I the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, as well as the possible benefits and have invited him/her to ask questions on any aspect of the study.

Signature, qualifications and date

Name in block capitals

4.2.3 Ethics Committee Approval Certificate

CONNOLLY HOSPITAL BLANCHARDSTOWN	
<u>Certificate of Research Ethics Committee Approval</u>	
Date:	8 th June, 2012
To:	Prof S Sreenan, Consultant Endocrinologist
From:	Dr Eamon Leen, Chairman
Protocol title:	The effect of Taurine on Endothelial Dysfunction in Patients with Type 2 Diabetes
<p>The Research Ethics Committee approved human subject involvement in your research project on 8th June, 2012.</p> <p>There is no expiration date for this approval, but the protocol must be reviewed by the Ethics Committee before December 31st 2014. If this project is to continue beyond that date, please submit an updated proposal one month prior to the expiration date. If this proposal is used in conjunction with any other human experimentation or if it is modified in any way, it must be re-approved for these special circumstances.</p> <p>Note that the following should be reported to the Ethics Committee: 1) all serious adverse events, occurring at this institution, regardless of whether or not they are thought to be study related should be reported within 2 business days to one of the members of the Research Ethics Committee, 2) any unanticipated problems, and/or 3) and injuries to subjects enrolled.</p> <p>Please remember that all data including all consent form documents must be returned for a minimum of three years past the completion of the research. Additional requirements may be imposed by your funding source, your department, or other entities. This institution protects personal health information of human subjects.</p>	
	
Dr Eamon Leen, Chairman	
Approval Period: 8 th June, 2012 - 31 st December 2014	



Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Connolly Hospital
Blanchardstown
Dublin 15

Tel: (01) 821 3844
Fax: (01) 646 5132

**Prof S Sreenan,
Consultant Endocrinologist,
Department of Endocrinology & Diabetes
Connolly Hospital**

12th June, 2012.

**Re: Study entitled The effect of Taurine on Endothelial
Dysfunction in Patients with Type 2 Diabetes**

Dear Prof Sreenan,

Thank you for submitting amendments to the above study which has now been approved.
Warmest regards,

Yours sincerely,

**Dr Eamon Leen,
Chairman,
Research Ethics Committee.**

4.2.4 Taurine and Placebo Documentation

LAMBERTS® ...at the leading edge of responsible nutrition		Lamberts Healthcare Ltd 1 Lamberts Road, Tunbridge Wells, Kent, TN2 3EH Telephone 01892 554312 Fax 01892 515863 www.lambertshealthcare.co.uk					
Dr Wan Aizad Wan Mahmood 1 Achill Square Waterville Blanchardstown Dublin 15 EIRE Ireland		Dr W A Wan Mahmood Diabetes Day Centre Connolly Hospital Blanchardstown Dublin 15 Ireland Ireland					
Page 1 of 1							
CUSTOMER NO.	ORDER NO.	ORDER DATE	DESPATCH DATE	METHOD	INVOICE NO.	DOCUMENT TYPE	
0005095714	1216922	07/09/2012	11/09/2012	AIRML	1209394	RECEIPT	
PRODUCT CODE	PICK LOC	QUANTITY		DESCRIPTION	UNIT PRICE	VAT CODE	AMOUNT
8325-60		ORD. 34	DESP. 34	Taurine 500mg (RRP £8.90)	4.97	STD	168.98
REMARKS 10% discount of order -16.90					GOODS		168.98
					POST & PACKING		15.20
VAT %	NETT GOODS P&P	VAT	PAYMENT	PAID/CHARGED	ORDER TOTAL	DISCOUNT	
20.00	167.28	33.45	MCARD	200.73	200.73	TOTAL	200.73

VAT No. GB 167 427 151

Registered in England under Co. No. 4500877

LAMBERTS®
 ...at the leading edge of responsible nutrition



This packaging is
100% recyclable.



**Practitioners
CHOICE** ✓

FREE TECHNICAL ADVICE

Return address: Lamberts Healthcare Ltd. 1 Lamberts Road, Tunbridge Wells, Kent, TN2 3EH.



Standard Operating Procedure Form Clinical Services - Quest Park

TITLE: QF301a – C/T Trial Information Sheet

Product Details – Product A

Please indicate drug type: **IMP**

Product:	Taurine	Brand name (if known, or if other state):	Taurine
Strength:	500mg	Pharmaceutical form:	Capsules
Preferred Pack Size:	60's	Country of Origin:	UK
Quantity required:	Mawdsleys not required to source as client sourcing product (please state if units or packs) * PLEASE NOTE: If Manufacture is required, we will increase the quantity to allow for damages etc.		
Batch requirements: (e.g. single batch)	Mawdsleys not required to source as client sourcing product	Expiry requirements: (e.g. 3 year expiry)	Mawdsleys not required to source as client sourcing product
Documentation required:	Mawdsleys not required to source as client sourcing product		

Product Details – Product B

Please indicate drug type: **Please select from drop down list:**

Product:	N/A	Brand name (if known, or if other state):	Empty bottles identical to placebo bottles
Strength:	N/A	Pharmaceutical form:	N/A
Preferred Pack Size:	N/A	Country of Origin:	N/A
Quantity required:	20 bottles (please state if units or packs) * PLEASE NOTE: If Manufacture is required, we will increase the quantity to allow for damages etc.		
Batch requirements: (e.g. single batch)	N/A	Expiry requirements: (e.g. 3 year expiry)	N/A
Documentation required:	N/A		

Product Details – Placebos

Preferred Placebo Material:	Microcrystalline Cellulose	Preferred pharmaceutical form:	Capsules
Preferred Pack Size:	100 capsules per bottle	Quantity of Placebos required:	2,000 capsules = 20 bottles * PLEASE NOTE: We will increase the quantity to account for production purposes etc.
Batch requirements: (e.g. single batch)	Single batch	Expiry requirements: (e.g. 3 year expiry)	1 year
Special requirements: (e.g. vegetarian)	Make sure does not contain any glucose as patients are diabetics		
Documentation required:	QP Certification		

I / We confirm that the information contained in the C/T Trial Information Sheet is correct and will apply to quotation version 2.0 when provided, and that the order will be subject to Mawdsleys standard trading terms as detailed in the Contract for Service.

Name:	Signature:	Date:
-------	------------	-------

Your enquiry will now be reviewed by Mawdsleys. After review we will issue the enquiry with an identifier and inform you of any comments etc, prior to creation and supply of a Provisional Quotation.

Written by: Kelly Ounsley Date: 04 th January 2012	Approved by: Date:
--	-----------------------

REVISION HISTORY		
Version	Revision	Supersedes
1.0	Initial version	New
2.0	Addition of further forms due to review of procedure	1.0
3.0	Review and streamline of form	2.0
4.0	Addition of further points identified during use of form.	3.0

5 References:

1. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001;89(1):E1-7.
2. Liew A, Barry F, O'Brien T. Endothelial progenitor cells: diagnostic and therapeutic considerations. *Bioessays*. 2006;28(3):261-70.
3. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*. 2006;368(9548):1696-705.
4. King TF, Bergin DA, Kent EM, Manning F, Reeves EP, Dicker P, et al. Endothelial progenitor cells in mothers of low-birthweight infants: a link between defective placental vascularization and increased cardiovascular risk? *J Clin Endocrinol Metab*. 2013;98(1):E33-9.
5. Laakso M. Cardiovascular disease in type 2 diabetes from population to man to mechanisms: the Kelly West Award Lecture 2008. *Diabetes Care*. 2010;33(2):442-9.
6. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *J Am Coll Cardiol*. 2010;55(13):1318-27.
7. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003;348(7):593-600.
8. Yamori Y, Liu L, Ikeda K, Miura A, Mizushima S, Miki T, et al. Distribution of twenty-four hour urinary taurine excretion and association with ischemic heart disease mortality in 24 populations of 16 countries: results from the WHO-CARDIAC study. *Hypertens Res*. 2001;24(4):453-7.
9. Ito T, Schaffer SW, Azuma J. The potential usefulness of taurine on diabetes mellitus and its complications. *Amino Acids*. 2012;42(5):1529-39.
10. Militante JD, Lombardini JB. Treatment of hypertension with oral taurine: experimental and clinical studies. *Amino Acids*. 2002;23(4):381-93.
11. Sun Q, Wang B, Li Y, Sun F, Li P, Xia W, et al. Taurine Supplementation Lowers Blood Pressure and Improves Vascular

Function in Prehypertension: Randomized, Double-Blind, Placebo-Controlled Study. *Hypertension*. 2016;67(3):541-9.

12. Choi MJ, Kim JH, Chang KJ. The effect of dietary taurine supplementation on plasma and liver lipid concentrations and free amino acid concentrations in rats fed a high-cholesterol diet. *Adv Exp Med Biol*. 2006;583:235-42.

13. Choi MJ, Kim MJ, Chang KJ. The effect of dietary taurine supplementation on plasma and liver lipid concentrations and mineral metabolism in rats fed alcohol. *Adv Exp Med Biol*. 2006;583:243-50.

14. Casey RG, Gang C, Joyce M, Bouchier-Hayes DJ. Taurine attenuates acute hyperglycaemia-induced endothelial cell apoptosis, leucocyte-endothelial cell interactions and cardiac dysfunction. *J Vasc Res*. 2007;44(1):31-9.

15. Wang LJ, Yu YH, Zhang LG, Wang Y, Niu N, Li Q, et al. Taurine rescues vascular endothelial dysfunction in streptozocin-induced diabetic rats: correlated with downregulation of LOX-1 and ICAM-1 expression on aortas. *Eur J Pharmacol*. 2008;597(1-3):75-80.

16. Moloney MA, Casey RG, DH OD, Fitzgerald P, Thompson C, Bouchier-Hayes DJ. Two weeks taurine supplementation reverses endothelial dysfunction in young male type 1 diabetics. *Diab Vasc Dis Res*. 2010.

17. Khakoo AY, Finkel T. Endothelial progenitor cells. *Annu Rev Med*. 2005;56:79-101.

18. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275(5302):964-7.

19. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. *Blood*. 1998;92(2):362-7.

20. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407(6801):249-57.

21. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med*. 1995;1(1):27-31.

22. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386(6626):671-4.

23. Rajasekar P, O'Neill CL, Eeles L, Stitt AW, Medina RJ. Epigenetic Changes in Endothelial Progenitors as a Possible Cellular Basis for Glycemic Memory in Diabetic Vascular Complications. *Journal of diabetes research*. 2015;2015:436879.
24. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circ Res*. 2012;110(4):624-37.
25. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res*. 2004;95(4):343-53.
26. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood*. 2000;95(10):3106-12.
27. Schmeisser A, Garlich CD, Zhang H, Eskafi S, Graffy C, Ludwig J, et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovasc Res*. 2001;49(3):671-80.
28. Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G, et al. Blood monocytes mimic endothelial progenitor cells. *Stem Cells*. 2006;24(2):357-67.
29. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation*. 2003;108(20):2511-6.
30. Prokopi M, Pula G, Mayr U, Devue C, Gallagher J, Xiao Q, et al. Proteomic analysis reveals presence of platelet microparticles in endothelial progenitor cell cultures. *Blood*. 2009;114(3):723-32.
31. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A*. 2000;97(7):3422-7.
32. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24(2):288-93.

33. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood*. 2007;109(5):1801-9.
34. Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C, Avogaro A. Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau. *Atherosclerosis*. 2008;197(2):496-503.
35. Sobrino T, Blanco M, Perez-Mato M, Rodriguez-Yanez M, Castillo J. Increased levels of circulating endothelial progenitor cells in patients with ischaemic stroke treated with statins during acute phase. *European journal of neurology : the official journal of the European Federation of Neurological Societies*. 2012;19(12):1539-46.
36. Lev EI, Leshem-Lev D, Mager A, Vaknin-Assa H, Harel N, Zimra Y, et al. Circulating endothelial progenitor cell levels and function in patients who experienced late coronary stent thrombosis. *Eur Heart J*. 2010;31(21):2625-32.
37. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest*. 2000;105(11):1527-36.
38. Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A, et al. Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. *Eur Heart J*. 2006;27(18):2247-55.
39. Vaughan EE, Liew A, Mashayekhi K, Dockery P, McDermott J, Kealy B, et al. Pretreatment of endothelial progenitor cells with osteopontin enhances cell therapy for peripheral vascular disease. *Cell Transplant*. 2012;21(6):1095-107.
40. Choi JH, Kim KL, Huh W, Kim B, Byun J, Suh W, et al. Decreased number and impaired angiogenic function of endothelial progenitor cells in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol*. 2004;24(7):1246-52.
41. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest*. 2001;108(3):391-7.
42. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation*. 2002;106(22):2781-6.

43. Griesse DP, Ehsan A, Melo LG, Kong D, Zhang L, Mann MJ, et al. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. *Circulation*. 2003;108(21):2710-5.
44. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation*. 2002;105(25):3017-24.
45. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res*. 2003;93(2):e17-24.
46. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med*. 2000;342(9):626-33.
47. Pillarisetti K, Gupta SK. Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1)1: SDF-1 alpha mRNA is selectively induced in rat model of myocardial infarction. *Inflammation*. 2001;25(5):293-300.
48. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res*. 2001;88(2):167-74.
49. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109(5):625-37.
50. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation*. 2001;103(23):2776-9.
51. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med*. 1999;5(4):434-8.
52. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, et al. Erythropoietin regulates endothelial progenitor cells. *Blood*. 2004;103(3):921-6.

53. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood*. 2003;102(4):1340-6.
54. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107(11):1395-402.
55. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. 2001;7(11):1194-201.
56. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res*. 2000;87(9):728-30.
57. De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med*. 2003;9(6):789-95.
58. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science*. 2003;300(5622):1155-9.
59. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest*. 2001;108(3):399-405.
60. Murayama T, Tepper OM, Silver M, Ma H, Losordo DW, Isner JM, et al. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp Hematol*. 2002;30(8):967-72.
61. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003;9(11):1370-6.
62. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107(8):1164-9.
63. Assmus B, Urbich C, Aicher A, Hofmann WK, Haendeler J, Rossig L, et al. HMG-CoA reductase inhibitors reduce senescence and increase

proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. *Circ Res*. 2003;92(9):1049-55.

64. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med*. 2000;6(9):1004-10.

65. Segal MS, Shah R, Afzal A, Perrault CM, Chang K, Schuler A, et al. Nitric oxide cytoskeletal-induced alterations reverse the endothelial progenitor cell migratory defect associated with diabetes. *Diabetes*. 2006;55(1):102-9.

66. Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation*. 2003;107(9):1322-8.

67. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med*. 2004;10(8):858-64.

68. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes: the role of reparatory mechanisms. *Diabetes Care*. 2011;34 Suppl 2:S285-90.

69. Floridi F, Trettel F, Di Bartolomeo S, Ciotti MT, Limatola C. Signalling pathways involved in the chemotactic activity of CXCL12 in cultured rat cerebellar neurons and CHP100 neuroepithelioma cells. *Journal of neuroimmunology*. 2003;135(1-2):38-46.

70. Imanishi T, Hano T, Nishio I. Estrogen reduces endothelial progenitor cell senescence through augmentation of telomerase activity. *J Hypertens*. 2005;23(9):1699-706.

71. Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J Immunol*. 1999;163(11):5954-63.

72. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1 α stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood*. 2000;95(8):2505-13.

73. Veit C, Genze F, Menke A, Hoeffert S, Gress TM, Gierschik P, et al. Activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase is required for glial cell line-derived neurotrophic factor-induced migration and invasion of pancreatic carcinoma cells. *Cancer Res.* 2004;64(15):5291-300.
74. Cheresh DA, Leng J, Klemke RL. Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *The Journal of cell biology.* 1999;146(5):1107-16.
75. Zheng H, Fu G, Dai T, Huang H. Migration of Endothelial Progenitor Cells Mediated by Stromal Cell-Derived Factor-1a/CXCR4 via PI3K/Akt/eNOS Signal Transduction Pathway. *J Cardiovasc Pharmacol.* 2007;50(3):274-80.
76. Wilson PW, Castelli WP, Kannel WB. Coronary risk prediction in adults (the Framingham Heart Study). *Am J Cardiol.* 1987;59(14):91G-4G.
77. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. *Hypertension.* 2010;55(5):1075-85.
78. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation.* 2005;111(22):2981-7.
79. Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol.* 2004;24(8):1442-7.
80. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8(4):403-9.
81. Imanishi T, Hano T, Matsuo Y, Nishio I. Oxidized low-density lipoprotein inhibits vascular endothelial growth factor-induced endothelial progenitor cell differentiation. *Clin Exp Pharmacol Physiol.* 2003;30(9):665-70.
82. Higashi Y, Matsuoka H, Umei H, Sugano R, Fujii Y, Soga J, et al. Endothelial function in subjects with isolated low HDL cholesterol: role of

nitric oxide and circulating progenitor cells. *Am J Physiol Endocrinol Metab.* 2010;298(2):E202-9.

83. Schmidt-Lucke C, Fichtlscherer S, Rossig L, Kamper U, Dimmeler S. Improvement of endothelial damage and regeneration indexes in patients with coronary artery disease after 4 weeks of statin therapy. *Atherosclerosis.* 2010;211(1):249-54.

84. Lin LY, Huang CC, Chen JS, Wu TC, Leu HB, Huang PH, et al. Effects of pitavastatin versus atorvastatin on the peripheral endothelial progenitor cells and vascular endothelial growth factor in high-risk patients: a pilot prospective, double-blind, randomized study. *Cardiovascular diabetology.* 2014;13(1):111.

85. Hibbert B, Simard T, Ramirez FD, Pourdjabbar A, Raizman JE, Maze R, et al. The effect of statins on circulating endothelial progenitor cells in humans: a systematic review. *J Cardiovasc Pharmacol.* 2013;62(5):491-6.

86. Reinhard H, Jacobsen PK, Lajer M, Pedersen N, Billestrup N, Mandrup-Poulsen T, et al. Multifactorial treatment increases endothelial progenitor cells in patients with type 2 diabetes. *Diabetologia.* 2010;53(10):2129-33.

87. Bahlmann FH, de Groot K, Mueller O, Hertel B, Haller H, Fliser D. Stimulation of endothelial progenitor cells: a new putative therapeutic effect of angiotensin II receptor antagonists. *Hypertension.* 2005;45(4):526-9.

88. Hu Z, Zhang F, Yang Z, Zhang J, Zhang D, Yang N, et al. Low-dose aspirin promotes endothelial progenitor cell migration and adhesion and prevents senescence. *Cell Biol Int.* 2008;32(7):761-8.

89. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med.* 1998;15(7):539-53.

90. Standards of medical care in diabetes--2014. *Diabetes Care.* 2014;37 Suppl 1:S14-80.

91. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet.* 2011;378(9785):31-40.

92. Balanda K FL, Jordan A, McArdle E. Making Diabetes Count. A Systematic Approach to Estimating Population Prevalence on the Island of Ireland in 2005. The Institute of Public Health in Ireland 2006. 2006.
93. Diabetes Briefing. The Institute of Public Health in Ireland 2012.
94. Wilson PW. Diabetes mellitus and coronary heart disease. *Am J Kidney Dis.* 1998;32(5 Suppl 3):S89-100.
95. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation.* 1998;97(18):1837-47.
96. McGill HC, Jr., McMahan CA. Determinants of atherosclerosis in the young. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *Am J Cardiol.* 1998;82(10B):30T-6T.
97. Stone PH, Muller JE, Hartwell T, York BJ, Rutherford JD, Parker CB, et al. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group. *J Am Coll Cardiol.* 1989;14(1):49-57.
98. Smith JW, Marcus FI, Serokman R. Prognosis of patients with diabetes mellitus after acute myocardial infarction. *Am J Cardiol.* 1984;54(7):718-21.
99. Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, Grishman A. New type of cardiomyopathy associated with diabetic glomerulosclerosis. *Am J Cardiol.* 1972;30(6):595-602.
100. Ettinger PO, Regan TJ. Cardiac disease in diabetes. *Postgraduate medicine.* 1989;85(6):229-32.
101. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care.* 1993;16(2):434-44.
102. Folsom AR, Eckfeldt JH, Weitzman S, Ma J, Chambless LE, Barnes RW, et al. Relation of carotid artery wall thickness to diabetes mellitus, fasting glucose and insulin, body size, and physical activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Stroke.* 1994;25(1):66-73.
103. O'Leary DH, Polak JF, Kronmal RA, Kittner SJ, Bond MG, Wolfson SK, Jr., et al. Distribution and correlates of sonographically detected

carotid artery disease in the Cardiovascular Health Study. The CHS Collaborative Research Group. *Stroke*. 1992;23(12):1752-60.

104. USRDS. USRDS 1994 Annual Data Report. Bethesda (MD), United States: National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, 1994. 1994.

105. McMillan MA, Briggs JD, Junor BJ. Outcome of renal replacement treatment in patients with diabetes mellitus. *BMJ*. 1990;301(6751):540-4.

106. Hirschl MM, Heinz G, Sunder-Plassmann G, Derfler K. Renal replacement therapy in type 2 diabetic patients: 10 years' experience. *Am J Kidney Dis*. 1992;20(6):564-8.

107. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med*. 2008;359(15):1577-89.

108. Holman RR, Sourij H, Califf RM. Cardiovascular outcome trials of glucose-lowering drugs or strategies in type 2 diabetes. *Lancet*. 2014;383(9933):2008-17.

109. American Diabetes A. 8. Cardiovascular Disease and Risk Management. *Diabetes Care*. 2016;39 Suppl 1:S60-71.

110. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. Management of hyperglycaemia in type 2 diabetes, 2015: a patient-centred approach. Update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetologia*. 2015;58(3):429-42.

111. American Diabetes A. (4) Foundations of care: education, nutrition, physical activity, smoking cessation, psychosocial care, and immunization. *Diabetes Care*. 2015;38 Suppl:S20-30.

112. American Diabetes A. (7) Approaches to glycemic treatment. *Diabetes Care*. 2015;38 Suppl:S41-8.

113. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet*. 1998;352(9131):854-65.

114. Schramm TK, Gislason GH, Vaag A, Rasmussen JN, Folke F, Hansen ML, et al. Mortality and cardiovascular risk associated with different insulin secretagogues compared with metformin in type 2

diabetes, with or without a previous myocardial infarction: a nationwide study. *Eur Heart J*. 2011;32(15):1900-8.

115. Johnson JA, Majumdar SR, Simpson SH, Toth EL. Decreased mortality associated with the use of metformin compared with sulfonylurea monotherapy in type 2 diabetes. *Diabetes Care*. 2002;25(12):2244-8.

116. Roumie CL, Hung AM, Greevy RA, Grijalva CG, Liu X, Murff HJ, et al. Comparative effectiveness of sulfonylurea and metformin monotherapy on cardiovascular events in type 2 diabetes mellitus: a cohort study. *Ann Intern Med*. 2012;157(9):601-10.

117. Hung YC, Lin CC, Wang TY, Chang MP, Sung FC, Chen CC. Oral hypoglycaemic agents and the development of non-fatal cardiovascular events in patients with type 2 diabetes mellitus. *Diabetes Metab Res Rev*. 2013;29(8):673-9.

118. Ferrannini E, DeFronzo RA. Impact of glucose-lowering drugs on cardiovascular disease in type 2 diabetes. *Eur Heart J*. 2015.

119. Hong J, Zhang Y, Lai S, Lv A, Su Q, Dong Y, et al. Effects of metformin versus glipizide on cardiovascular outcomes in patients with type 2 diabetes and coronary artery disease. *Diabetes Care*. 2013;36(5):1304-11.

120. Katakami N, Yamasaki Y, Hayaishi-Okano R, Ohtoshi K, Kaneto H, Matsuhisa M, et al. Metformin or gliclazide, rather than glibenclamide, attenuate progression of carotid intima-media thickness in subjects with type 2 diabetes. *Diabetologia*. 2004;47(11):1906-13.

121. Meaney E, Vela A, Samaniego V, Meaney A, Asbun J, Zempoalteca JC, et al. Metformin, arterial function, intima-media thickness and nitrooxidation in metabolic syndrome: the mefisto study. *Clin Exp Pharmacol Physiol*. 2008;35(8):895-903.

122. Matsumoto K, Sera Y, Abe Y, Tominaga T, Yeki Y, Miyake S. Metformin attenuates progression of carotid arterial wall thickness in patients with type 2 diabetes. *Diabetes Res Clin Pract*. 2004;64(3):225-8.

123. Preiss D, Lloyd SM, Ford I, McMurray JJ, Holman RR, Welsh P, et al. Metformin for non-diabetic patients with coronary heart disease (the CAMERA study): a randomised controlled trial. *Lancet Diabetes Endocrinol*. 2014;2(2):116-24.

124. Nagi DK, Yudkin JS. Effects of metformin on insulin resistance, risk factors for cardiovascular disease, and plasminogen activator

inhibitor in NIDDM subjects. A study of two ethnic groups. *Diabetes Care*. 1993;16(4):621-9.

125. Singh S, Bhat J, Wang PH. Cardiovascular effects of anti-diabetic medications in type 2 diabetes mellitus. *Current cardiology reports*. 2013;15(1):327.

126. Goldner MG, Knatterud GL, Prout TE. Effects of hypoglycemic agents on vascular complications in patients with adult-onset diabetes. 3. Clinical implications of UGDP results. *JAMA*. 1971;218(9):1400-10.

127. Rosenstock J, Marx N, Kahn SE, Zinman B, Kastelein JJ, Lachin JM, et al. Cardiovascular outcome trials in type 2 diabetes and the sulphonylurea controversy: rationale for the active-comparator CAROLINA trial. *Diab Vasc Dis Res*. 2013;10(4):289-301.

128. Tomai F, Crea F, Gaspardone A, Versaci F, De Paulis R, Penta de Peppo A, et al. Ischemic preconditioning during coronary angioplasty is prevented by glibenclamide, a selective ATP-sensitive K⁺ channel blocker. *Circulation*. 1994;90(2):700-5.

129. Klepzig H, Kober G, Matter C, Luus H, Schneider H, Boedeker KH, et al. Sulfonylureas and ischaemic preconditioning; a double-blind, placebo-controlled evaluation of glimepiride and glibenclamide. *Eur Heart J*. 1999;20(6):439-46.

130. Zeller M, Danchin N, Simon D, Vahanian A, Lorgis L, Cottin Y, et al. Impact of type of preadmission sulfonylureas on mortality and cardiovascular outcomes in diabetic patients with acute myocardial infarction. *J Clin Endocrinol Metab*. 2010;95(11):4993-5002.

131. Evans JM, Ogston SA, Emslie-Smith A, Morris AD. Risk of mortality and adverse cardiovascular outcomes in type 2 diabetes: a comparison of patients treated with sulfonylureas and metformin. *Diabetologia*. 2006;49(5):930-6.

132. Rao AD, Kuhadiya N, Reynolds K, Fonseca VA. Is the combination of sulfonylureas and metformin associated with an increased risk of cardiovascular disease or all-cause mortality?: a meta-analysis of observational studies. *Diabetes Care*. 2008;31(8):1672-8.

133. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet*. 1998;352(9131):837-53.

134. Landman GW, de Bock GH, van Hateren KJ, van Dijk PR, Groenier KH, Gans RO, et al. Safety and efficacy of gliclazide as treatment for type 2 diabetes: a systematic review and meta-analysis of randomized trials. *PLoS One*. 2014;9(2):e82880.
135. Sadikot SM, Mogensen CE. Risk of coronary artery disease associated with initial sulphonylurea treatment of patients with type 2 diabetes: a matched case-control study. *Diabetes Res Clin Pract*. 2008;82(3):391-5.
136. Jorgensen CH, Gislason GH, Andersson C, Ahlehoff O, Charlot M, Schramm TK, et al. Effects of oral glucose-lowering drugs on long term outcomes in patients with diabetes mellitus following myocardial infarction not treated with emergent percutaneous coronary intervention--a retrospective nationwide cohort study. *Cardiovascular diabetology*. 2010;9:54.
137. Vamecq J, Latruffe N. Medical significance of peroxisome proliferator-activated receptors. *Lancet*. 1999;354(9173):141-8.
138. Haffner SM. Epidemiology of hypertension and insulin resistance syndrome. *J Hypertens Suppl*. 1997;15(1):S25-30.
139. Qayyum R, Adomaityte J. A meta-analysis of the effect of thiazolidinediones on blood pressure. *Journal of clinical hypertension*. 2006;8(1):19-28.
140. Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW, et al. The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes*. 2002;51(3):797-802.
141. Goldberg RB, Kendall DM, Deeg MA, Buse JB, Zagar AJ, Pinaire JA, et al. A comparison of lipid and glycemic effects of pioglitazone and rosiglitazone in patients with type 2 diabetes and dyslipidemia. *Diabetes Care*. 2005;28(7):1547-54.
142. Robinson JG. Should We Use PPAR Agonists to Reduce Cardiovascular Risk? *PPAR Res*. 2008;2008:891425.
143. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, et al. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation*. 2003;108(23):2941-8.

144. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med*. 2007;356(24):2457-71.
145. Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M, et al. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet*. 2009;373(9681):2125-35.
146. Hiatt WR, Kaul S, Smith RJ. The cardiovascular safety of diabetes drugs--insights from the rosiglitazone experience. *N Engl J Med*. 2013;369(14):1285-7.
147. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet*. 2005;366(9493):1279-89.
148. Dormandy J, Bhattacharya M, van Troostenburg de Bruyn AR, investigators PR. Safety and tolerability of pioglitazone in high-risk patients with type 2 diabetes: an overview of data from PROactive. *Drug Saf*. 2009;32(3):187-202.
149. Ferwana M, Firwana B, Hasan R, Al-Mallah MH, Kim S, Montori VM, et al. Pioglitazone and risk of bladder cancer: a meta-analysis of controlled studies. *Diabet Med*. 2013;30(9):1026-32.
150. FDA. Guidance for Industry: diabetes mellitus - evaluating cardiovascular risk in new antidiabetic therapies to treat type 2 diabetes. 2008.
151. Chia CW, Egan JM. Incretin-based therapies in type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2008;93(10):3703-16.
152. Frederich R, Alexander JH, Fiedorek FT, Donovan M, Berglind N, Harris S, et al. A systematic assessment of cardiovascular outcomes in the saxagliptin drug development program for type 2 diabetes. *Postgraduate medicine*. 2010;122(3):16-27.
153. Monami M, Iacomelli I, Marchionni N, Mannucci E. Dipeptidyl peptidase-4 inhibitors in type 2 diabetes: a meta-analysis of randomized clinical trials. *Nutr Metab Cardiovasc Dis*. 2010;20(4):224-35.

154. White WB, Cannon CP, Heller SR, Nissen SE, Bergenstal RM, Bakris GL, et al. Alogliptin after acute coronary syndrome in patients with type 2 diabetes. *N Engl J Med*. 2013;369(14):1327-35.
155. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med*. 2013;369(14):1317-26.
156. Haffner SM. Pre-diabetes, insulin resistance, inflammation and CVD risk. *Diabetes Res Clin Pract*. 2003;61 Suppl 1:S9-S18.
157. Hadi HA, Suwaidi JA. Endothelial dysfunction in diabetes mellitus. *Vasc Health Risk Manag*. 2007;3(6):853-76.
158. Boden G, Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *Eur J Clin Invest*. 2002;32 Suppl 3:14-23.
159. Lyon CJ, Law RE, Hsueh WA. Minireview: adiposity, inflammation, and atherogenesis. *Endocrinology*. 2003;144(6):2195-200.
160. Hsueh WA, Lyon CJ, Quinones MJ. Insulin resistance and the endothelium. *Am J Med*. 2004;117(2):109-17.
161. Okouchi M, Okayama N, Imai S, Omi H, Shimizu M, Fukutomi T, et al. High insulin enhances neutrophil transendothelial migration through increasing surface expression of platelet endothelial cell adhesion molecule-1 via activation of mitogen activated protein kinase. *Diabetologia*. 2002;45(10):1449-56.
162. Hartge MM, Unger T, Kintscher U. The endothelium and vascular inflammation in diabetes. *Diab Vasc Dis Res*. 2007;4(2):84-8.
163. Piconi L, Quagliaro L, Assaloni R, Da Ros R, Maier A, Zuodar G, et al. Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction. *Diabetes Metab Res Rev*. 2006;22(3):198-203.
164. Liu XF, Yu JQ, Dalan R, Liu AQ, Luo KQ. Biological factors in plasma from diabetes mellitus patients enhance hyperglycaemia and pulsatile shear stress-induced endothelial cell apoptosis. *Integr Biol (Camb)*. 2014;6(5):511-22.
165. McClung JA, Naseer N, Saleem M, Rossi GP, Weiss MB, Abraham NG, et al. Circulating endothelial cells are elevated in patients with type 2 diabetes mellitus independently of HbA(1)c. *Diabetologia*. 2005;48(2):345-50.

166. Nomura S. Dynamic role of microparticles in type 2 diabetes mellitus. *Curr Diabetes Rev.* 2009;5(4):245-51.
167. Werner N, Nickenig G. Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? *Arterioscler Thromb Vasc Biol.* 2006;26(2):257-66.
168. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol.* 2005;45(9):1449-57.
169. Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes.* 2004;53(1):195-9.
170. Fadini GP, Boscaro E, de Kreutzenberg S, Agostini C, Seeger F, Dimmeler S, et al. Time course and mechanisms of circulating progenitor cell reduction in the natural history of type 2 diabetes. *Diabetes Care.* 2010;33(5):1097-102.
171. Fadini GP, Sartore S, Albiero M, Baesso I, Murphy E, Menegolo M, et al. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. *Arterioscler Thromb Vasc Biol.* 2006;26(9):2140-6.
172. Fadini GP, Sartore S, Agostini C, Avogaro A. Significance of endothelial progenitor cells in subjects with diabetes. *Diabetes Care.* 2007;30(5):1305-13.
173. Sutter CH, Laughner E, Semenza GL. Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. *Proc Natl Acad Sci U S A.* 2000;97(9):4748-53.
174. Fadini GP, Sartore S, Schiavon M, Albiero M, Baesso I, Cabrelle A, et al. Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia.* 2006;49(12):3075-84.
175. Ebrahimian TG, Heymes C, You D, Blanc-Brude O, Mees B, Waeckel L, et al. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol.* 2006;169(2):719-28.
176. Humpert PM, Neuwirth R, Battista MJ, Voronko O, von Eynatten M, Konrade I, et al. SDF-1 genotype influences insulin-dependent

mobilization of adult progenitor cells in type 2 diabetes. *Diabetes Care*. 2005;28(4):934-6.

177. Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA*. 2002;288(20):2579-88.

178. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*. 2005;54(6):1615-25.

179. Sambucetti G, Morbelli S, Vanella L, Kusmic C, Marini C, Massollo M, et al. Diabetes impairs the vascular recruitment of normal stem cells by oxidant damage, reversed by increases in pAMPK, heme oxygenase-1, and adiponectin. *Stem Cells*. 2009;27(2):399-407.

180. Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, et al. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation*. 2004;109(17):2058-67.

181. Fadini GP, Schiavon M, Cantini M, Baesso I, Facco M, Miorin M, et al. Circulating progenitor cells are reduced in patients with severe lung disease. *Stem Cells*. 2006;24(7):1806-13.

182. Avogaro A, Pagnin E, Calo L. Monocyte NADPH oxidase subunit p22(phox) and inducible hemeoxygenase-1 gene expressions are increased in type II diabetic patients: relationship with oxidative stress. *J Clin Endocrinol Metab*. 2003;88(4):1753-9.

183. Krankel N, Adams V, Linke A, Gielen S, Erbs S, Lenk K, et al. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. *Arterioscler Thromb Vasc Biol*. 2005;25(4):698-703.

184. Fadini GP, Boscaro E, Albiero M, Menegazzo L, Frison V, de Kreutzenberg S, et al. The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha. *Diabetes Care*. 2010;33(7):1607-9.

185. Poncina N, Albiero M, Menegazzo L, Cappellari R, Avogaro A, Fadini GP. The dipeptidyl peptidase-4 inhibitor saxagliptin improves function of circulating pro-angiogenic cells from type 2 diabetic patients. *Cardiovascular diabetology*. 2014;13:92.

186. Fadini GP, Albiero M, Menegazzo L, de Kreutzenberg SV, Avogaro A. The increased dipeptidyl peptidase-4 activity is not counteracted by

optimized glucose control in type 2 diabetes, but is lower in metformin-treated patients. *Diabetes Obes Metab.* 2012;14(6):518-22.

187. Chen LL, Liao YF, Zeng TS, Yu F, Li HQ, Feng Y. Effects of metformin plus gliclazide compared with metformin alone on circulating endothelial progenitor cell in type 2 diabetic patients. *Endocrine.* 2010;38(2):266-75.

188. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* 2001;108(8):1167-74.

189. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes.* 2002;51(7):2074-81.

190. Kao J, Tobis J, McClelland RL, Heaton MR, Davis BR, Holmes DR, Jr., et al. Relation of metformin treatment to clinical events in diabetic patients undergoing percutaneous intervention. *Am J Cardiol.* 2004;93(11):1347-50, A5.

191. Chen LL, Yu F, Zeng TS, Liao YF, Li YM, Ding HC. Effects of gliclazide on endothelial function in patients with newly diagnosed type 2 diabetes. *Eur J Pharmacol.* 2011;659(2-3):296-301.

192. Esposito K, Maiorino MI, Di Palo C, Gicchino M, Petrizzo M, Bellastella G, et al. Effects of pioglitazone versus metformin on circulating endothelial microparticles and progenitor cells in patients with newly diagnosed type 2 diabetes--a randomized controlled trial. *Diabetes Obes Metab.* 2011;13(5):439-45.

193. Matsuo Y, Imanishi T, Kuroi A, Kitabata H, Kubo T, Hayashi Y, et al. Effects of plasma adiponectin levels on the number and function of endothelial progenitor cells in patients with coronary artery disease. *Circ J.* 2007;71(9):1376-82.

194. Wang CH, Ting MK, Verma S, Kuo LT, Yang NI, Hsieh IC, et al. Pioglitazone increases the numbers and improves the functional capacity of endothelial progenitor cells in patients with diabetes mellitus. *Am Heart J.* 2006;152(6):1051 e1-8.

195. Makino H, Okada S, Nagumo A, Sugisawa T, Miyamoto Y, Kishimoto I, et al. Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients. *Diabetes Res Clin Pract.* 2008;81(3):327-30.

196. Tomlinson LA. Methods for assessing arterial stiffness: technical considerations. *Curr Opin Nephrol Hypertens*. 2012;21(6):655-60.
197. Prenner SB, Chirinos JA. Arterial stiffness in diabetes mellitus. *Atherosclerosis*. 2015;238(2):370-9.
198. Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. *Eur Heart J*. 2006;27(21):2588-605.
199. Frimodt-Moller M, Nielsen AH, Kamper AL, Strandgaard S. Reproducibility of pulse-wave analysis and pulse-wave velocity determination in chronic kidney disease. *Nephrol Dial Transplant*. 2008;23(2):594-600.
200. Pannier B, Guerin AP, Marchais SJ, Safar ME, London GM. Stiffness of capacitive and conduit arteries: prognostic significance for end-stage renal disease patients. *Hypertension*. 2005;45(4):592-6.
201. Van Bortel LM, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank JK, De Backer T, et al. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *J Hypertens*. 2012;30(3):445-8.
202. Reference Values for Arterial Stiffness C. Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: 'establishing normal and reference values'. *Eur Heart J*. 2010;31(19):2338-50.
203. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J*. 2007;28(12):1462-536.
204. McEniery CM, Yasmin, Hall IR, Qasem A, Wilkinson IB, Cockcroft JR, et al. Normal vascular aging: differential effects on wave reflection and aortic pulse wave velocity: the Anglo-Cardiff Collaborative Trial (ACCT). *J Am Coll Cardiol*. 2005;46(9):1753-60.
205. Cecelja M, Chowienczyk P. Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: a systematic review. *Hypertension*. 2009;54(6):1328-36.

206. Kimoto E, Shoji T, Shinohara K, Hatsuda S, Mori K, Fukumoto S, et al. Regional arterial stiffness in patients with type 2 diabetes and chronic kidney disease. *J Am Soc Nephrol*. 2006;17(8):2245-52.
207. Cameron JD, Bulpitt CJ, Pinto ES, Rajkumar C. The aging of elastic and muscular arteries: a comparison of diabetic and nondiabetic subjects. *Diabetes Care*. 2003;26(7):2133-8.
208. Webb DR, Khunti K, Silverman R, Gray LJ, Srinivasan B, Lacy PS, et al. Impact of metabolic indices on central artery stiffness: independent association of insulin resistance and glucose with aortic pulse wave velocity. *Diabetologia*. 2010;53(6):1190-8.
209. Safar ME, Czernichow S, Blacher J. Obesity, arterial stiffness, and cardiovascular risk. *J Am Soc Nephrol*. 2006;17(4 Suppl 2):S109-11.
210. Nakanishi N, Suzuki K, Tatara K. Clustered features of the metabolic syndrome and the risk for increased aortic pulse wave velocity in middle-aged Japanese men. *Angiology*. 2003;54(5):551-9.
211. Dinneen SF, Gerstein HC. The association of microalbuminuria and mortality in non-insulin-dependent diabetes mellitus. A systematic overview of the literature. *Arch Intern Med*. 1997;157(13):1413-8.
212. Molitch ME, Steffes M, Sun W, Rutledge B, Cleary P, de Boer IH, et al. Development and progression of renal insufficiency with and without albuminuria in adults with type 1 diabetes in the diabetes control and complications trial and the epidemiology of diabetes interventions and complications study. *Diabetes Care*. 2010;33(7):1536-43.
213. Smith A, Karalliedde J, De Angelis L, Goldsmith D, Viberti G. Aortic pulse wave velocity and albuminuria in patients with type 2 diabetes. *J Am Soc Nephrol*. 2005;16(4):1069-75.
214. Ishimura E, Taniwaki H, Tsuchida T, Obatake N, Emoto M, Shoji T, et al. Urinary albumin excretion associated with arterial wall stiffness rather than thickness in type 2 diabetic patients. *J Nephrol*. 2007;20(2):204-11.
215. Targher G, Bertolini L, Zenari L, Lippi G, Pichiri I, Zoppini G, et al. Diabetic retinopathy is associated with an increased incidence of cardiovascular events in Type 2 diabetic patients. *Diabet Med*. 2008;25(1):45-50.
216. Kim WJ, Park CY, Park SE, Rhee EJ, Lee WY, Oh KW, et al. The association between regional arterial stiffness and diabetic retinopathy in type 2 diabetes. *Atherosclerosis*. 2012;225(1):237-41.

217. Chen HS, Hwu CM, Kuo BI, Chiang SC, Kwok CF, Lee SH, et al. Abnormal cardiovascular reflex tests are predictors of mortality in Type 2 diabetes mellitus. *Diabet Med*. 2001;18(4):268-73.
218. Meyer C, Milat F, McGrath BP, Cameron J, Kotsopoulos D, Teede HJ. Vascular dysfunction and autonomic neuropathy in Type 2 diabetes. *Diabet Med*. 2004;21(7):746-51.
219. Theilade S, Lajer M, Persson F, Joergensen C, Rossing P. Arterial stiffness is associated with cardiovascular, renal, retinal, and autonomic disease in type 1 diabetes. *Diabetes Care*. 2013;36(3):715-21.
220. Debette S, Markus HS. The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. *BMJ*. 2010;341:c3666.
221. Laugesen E, Hoyem P, Stausbol-Gron B, Mikkelsen A, Thrysoe S, Erlandsen M, et al. Carotid-femoral pulse wave velocity is associated with cerebral white matter lesions in type 2 diabetes. *Diabetes Care*. 2013;36(3):722-8.
222. Palombo C, Kozakova M, Morizzo C, Gnesi L, Barsotti MC, Spontoni P, et al. Circulating endothelial progenitor cells and large artery structure and function in young subjects with uncomplicated type 1 diabetes. *Cardiovascular diabetology*. 2011;10:88.
223. Yue WS, Lau KK, Siu CW, Wang M, Yan GH, Yiu KH, et al. Impact of glycemic control on circulating endothelial progenitor cells and arterial stiffness in patients with type 2 diabetes mellitus. *Cardiovascular diabetology*. 2011;10:113.
224. Protosaltis I, Foussas S, Angelidi A, Gritzapis A, Sergeantanis T, Matsagos S, et al. Impact of ADMA, endothelial progenitor cells and traditional cardiovascular risk factors on pulse wave velocity among prediabetic individuals. *Cardiovascular diabetology*. 2012;11:141.
225. Huxtable RJ. Physiological actions of taurine. *Physiol Rev*. 1992;72(1):101-63.
226. Hayes KC, Rabin AR, Berson EL. An ultrastructural study of nutritionally induced and reversed retinal degeneration in cats. *Am J Pathol*. 1975;78(3):505-24.
227. Hayes KC, Sturman JA. Taurine in metabolism. *Annu Rev Nutr*. 1981;1:401-25.

228. Bankson DD, Russell RM. Protein energy malnutrition and taurine supplementation: effects on vitamin A nutritional status and electroretinogram of young rats. *J Nutr.* 1988;118(1):23-32.
229. Satoh H. Cardiac actions of taurine as a modulator of the ion channels. *Adv Exp Med Biol.* 1998;442:121-8.
230. Schaffer S, Takahashi K, Azuma J. Role of osmoregulation in the actions of taurine. *Amino Acids.* 2000;19(3-4):527-46.
231. Sjovall J. Dietary glycine and taurine on bile acid conjugation in man; bile acids and steroids 75. *Proc Soc Exp Biol Med.* 1959;100(4):676-8.
232. Weiss SJ, Klein R, Slivka A, Wei M. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J Clin Invest.* 1982;70(3):598-607.
233. Pennathur S, Heinecke JW. Oxidative stress and endothelial dysfunction in vascular disease. *Curr Diab Rep.* 2007;7(4):257-64.
234. Omi H, Okayama N, Shimizu M, Okouchi M, Ito S, Fukutomi T, et al. Participation of high glucose concentrations in neutrophil adhesion and surface expression of adhesion molecules on cultured human endothelial cells: effect of antidiabetic medicines. *Journal of diabetes and its complications.* 2002;16(3):201-8.
235. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, et al. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA.* 2001;286(17):2136-42.
236. Ogasawara M, Nakamura T, Koyama I, Nemoto M, Yoshida T. Reactivity of taurine with aldehydes and its physiological role. *Chem Pharm Bull (Tokyo).* 1993;41(12):2172-5.
237. Hu J, Xu X, Yang J, Wu G, Sun C, Lv Q. Antihypertensive effect of taurine in rat. *Adv Exp Med Biol.* 2009;643:75-84.
238. Selvaraj N, Bobby Z, Sathiyapriya V. Effect of lipid peroxides and antioxidants on glycation of hemoglobin: an in vitro study on human erythrocytes. *Clin Chim Acta.* 2006;366(1-2):190-5.
239. Nandhini AT, Thirunavukkarasu V, Anuradha CV. Stimulation of glucose utilization and inhibition of protein glycation and AGE products by taurine. *Acta Physiol Scand.* 2004;181(3):297-303.

240. Schaffer SW, Azuma J, Mozaffari M. Role of antioxidant activity of taurine in diabetes. *Canadian journal of physiology and pharmacology*. 2009;87(2):91-9.
241. Sturman JA, Hepner GW, Hofmann AF, Thomas PJ. Metabolism of [35S]taurine in man. *J Nutr*. 1975;105(9):1206-14.
242. Brons C, Spohr C, Storgaard H, Dyerberg J, Vaag A. Effect of taurine treatment on insulin secretion and action, and on serum lipid levels in overweight men with a genetic predisposition for type II diabetes mellitus. *Eur J Clin Nutr*. 2004;58(9):1239-47.
243. Alvarado-Vasquez N, Zamudio P, Ceron E, Vanda B, Zenteno E, Carvajal-Sandoval G. Effect of glycine in streptozotocin-induced diabetic rats. *Comp Biochem Physiol C Toxicol Pharmacol*. 2003;134(4):521-7.
244. Di Leo MA, Santini SA, Silveri NG, Giardina B, Franconi F, Ghirlanda G. Long-term taurine supplementation reduces mortality rate in streptozotocin-induced diabetic rats. *Amino Acids*. 2004;27(2):187-91.
245. Arany E, Strutt B, Romanus P, Remacle C, Reusens B, Hill DJ. Taurine supplement in early life altered islet morphology, decreased insulinitis and delayed the onset of diabetes in non-obese diabetic mice. *Diabetologia*. 2004;47(10):1831-7.
246. Harada N, Ninomiya C, Osako Y, Morishima M, Mawatari K, Takahashi A, et al. Taurine alters respiratory gas exchange and nutrient metabolism in type 2 diabetic rats. *Obes Res*. 2004;12(7):1077-84.
247. Nakaya Y, Minami A, Harada N, Sakamoto S, Niwa Y, Ohnaka M. Taurine improves insulin sensitivity in the Otsuka Long-Evans Tokushima Fatty rat, a model of spontaneous type 2 diabetes. *Am J Clin Nutr*. 2000;71(1):54-8.
248. El Mesallamy HO, El-Demerdash E, Hammad LN, El Magdoub HM. Effect of taurine supplementation on hyperhomocysteinemia and markers of oxidative stress in high fructose diet induced insulin resistance. *Diabetol Metab Syndr*. 2010;2:46.
249. Huang JS, Chuang LY, Guh JY, Huang YJ, Hsu MS. Antioxidants attenuate high glucose-induced hypertrophic growth in renal tubular epithelial cells. *Am J Physiol Renal Physiol*. 2007;293(4):F1072-82.
250. Yao HT, Lin P, Chang YW, Chen CT, Chiang MT, Chang L, et al. Effect of taurine supplementation on cytochrome P450 2E1 and oxidative stress in the liver and kidneys of rats with streptozotocin-induced diabetes. *Food Chem Toxicol*. 2009;47(7):1703-9.

251. Huang JS, Chuang LY, Guh JY, Huang YJ. Effects of nitric oxide and antioxidants on advanced glycation end products-induced hypertrophic growth in human renal tubular cells. *Toxicol Sci.* 2009;111(1):109-19.
252. Zeng K, Xu H, Mi M, Zhang Q, Zhang Y, Chen K, et al. Dietary taurine supplementation prevents glial alterations in retina of diabetic rats. *Neurochem Res.* 2009;34(2):244-54.
253. Zeng K, Xu H, Chen K, Zhu J, Zhou Y, Zhang Q, et al. Effects of taurine on glutamate uptake and degradation in Muller cells under diabetic conditions via antioxidant mechanism. *Mol Cell Neurosci.* 2010;45(2):192-9.
254. Li F, Obrosova IG, Abatan O, Tian D, Larkin D, Stuenkel EL, et al. Taurine replacement attenuates hyperalgesia and abnormal calcium signaling in sensory neurons of STZ-D rats. *Am J Physiol Endocrinol Metab.* 2005;288(1):E29-36.
255. Franconi F, Bennardini F, Mattana A, Miceli M, Ciuti M, Mian M, et al. Plasma and platelet taurine are reduced in subjects with insulin-dependent diabetes mellitus: effects of taurine supplementation. *Am J Clin Nutr.* 1995;61(5):1115-9.
256. Franconi F, Miceli M, Fazzini A, Seghieri G, Caputo S, DiLeo MA, et al. Taurine and diabetes. Humans and experimental models. *Adv Exp Med Biol.* 1996;403:579-82.
257. De Luca G, Calpona PR, Caponetti A, Romano G, Di Benedetto A, Cucinotta D, et al. Taurine and osmoregulation: platelet taurine content, uptake, and release in type 2 diabetic patients. *Metabolism.* 2001;50(1):60-4.
258. Chauncey KB, Tenner TE, Jr., Lombardini JB, Jones BG, Brooks ML, Warner RD, et al. The effect of taurine supplementation on patients with type 2 diabetes mellitus. *Adv Exp Med Biol.* 2003;526:91-6.
259. Elizarova EP, Nedosugova LV. First experiments in taurine administration for diabetes mellitus. The effect on erythrocyte membranes. *Adv Exp Med Biol.* 1996;403:583-8.
260. Xiao C, Giacca A, Lewis GF. Oral taurine but not N-acetylcysteine ameliorates NEFA-induced impairment in insulin sensitivity and beta cell function in obese and overweight, non-diabetic men. *Diabetologia.* 2008;51(1):139-46.

261. Hansen SH. The role of taurine in diabetes and the development of diabetic complications. *Diabetes Metab Res Rev*. 2001;17(5):330-46.
262. Casey RG, Joyce M, Moore K, Thompson C, Fitzgerald P, Bouchier-Hayes DJ. Two-week treatment with pravastatin improves ventriculo-vascular haemodynamic interactions in young men with type 1 diabetes. *Diab Vasc Dis Res*. 2007;4(1):53-61.
263. Fennessy FM, Moneley DS, Wang JH, Kelly CJ, Bouchier-Hayes DJ. Taurine and vitamin C modify monocyte and endothelial dysfunction in young smokers. *Circulation*. 2003;107(3):410-5.
264. Chen JZ, Zhang FR, Tao QM, Wang XX, Zhu JH. Number and activity of endothelial progenitor cells from peripheral blood in patients with hypercholesterolaemia. *Clin Sci (Lond)*. 2004;107(3):273-80.
265. Michaud SE, Dussault S, Haddad P, Groleau J, Rivard A. Circulating endothelial progenitor cells from healthy smokers exhibit impaired functional activities. *Atherosclerosis*. 2006;187(2):423-32.
266. Liew A, McDermott JH, Barry F, O'Brien T. Endothelial progenitor cells for the treatment of diabetic vasculopathy: panacea or Pandora's box? *Diabetes Obes Metab*. 2008;10(5):353-66.
267. Di Stefano R, Barsotti MC, Felice F, Magera A, Lekakis J, Leone A, et al. Smoking and endothelial progenitor cells: a revision of literature. *Current pharmaceutical design*. 2010;16(23):2559-66.
268. Scirica BM, Braunwald E, Raz I, Cavender MA, Morrow DA, Jarolim P, et al. Heart failure, saxagliptin, and diabetes mellitus: observations from the SAVOR-TIMI 53 randomized trial. *Circulation*. 2014;130(18):1579-88.
269. Anderson TJ. Arterial stiffness or endothelial dysfunction as a surrogate marker of vascular risk. *The Canadian journal of cardiology*. 2006;22 Suppl B:72B-80B.
270. Yamori Y, Taguchi T, Hamada A, Kunimasa K, Mori H, Mori M. Taurine in health and diseases: consistent evidence from experimental and epidemiological studies. *J Biomed Sci*. 2010;17 Suppl 1:S6.
271. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol*. 2002;39(2):257-65.

272. Hickson SS, Butlin M, Broad J, Avolio AP, Wilkinson IB, McEniery CM. Validity and repeatability of the Vicorder apparatus: a comparison with the SphygmoCor device. *Hypertens Res.* 2009;32(12):1079-85.
273. Kis E, Cseprekal O, Kerti A, Salvi P, Benetos A, Tisler A, et al. Measurement of pulse wave velocity in children and young adults: a comparative study using three different devices. *Hypertens Res.* 2011;34(11):1197-202.
274. Kracht D, Shroff R, Baig S, Doyon A, Jacobi C, Zeller R, et al. Validating a new oscillometric device for aortic pulse wave velocity measurements in children and adolescents. *Am J Hypertens.* 2011;24(12):1294-9.
275. Pucci G, Cheriyan J, Hubsch A, Hickson SS, Gajendragadkar PR, Watson T, et al. Evaluation of the Vicorder, a novel cuff-based device for the noninvasive estimation of central blood pressure. *J Hypertens.* 2013;31(1):77-85.
276. Kolcak J, Stuchlik M, Rabiskova M. [Soft gelatin capsules, their manufacture and evaluation]. *Ceska Slov Farm.* 2003;52(1):4-11.
277. Rogers TL, Wallick D. Reviewing the use of ethylcellulose, methylcellulose and hypromellose in microencapsulation. Part 1: materials used to formulate microcapsules. *Drug Dev Ind Pharm.* 2012;38(2):129-57.
278. Nakamura T, Ushiyama C, Suzuki S, Shimada N, Ohmuro H, Ebihara I, et al. Effects of taurine and vitamin E on microalbuminuria, plasma metalloproteinase-9, and serum type IV collagen concentrations in patients with diabetic nephropathy. *Nephron.* 1999;83(4):361-2.
279. Ohnishi H, Saitoh S, Takagi S, Ohata J, Isobe T, Kikuchi Y, et al. Pulse wave velocity as an indicator of atherosclerosis in impaired fasting glucose: the Tanno and Sobetsu study. *Diabetes Care.* 2003;26(2):437-40.
280. Schram MT, Henry RM, van Dijk RA, Kostense PJ, Dekker JM, Nijpels G, et al. Increased central artery stiffness in impaired glucose metabolism and type 2 diabetes: the Hoorn Study. *Hypertension.* 2004;43(2):176-81.
281. Fang FS, Liu MY, Cheng XL, Zhong WW, Miao XY, Li J, et al. Insulin resistance correlates with the arterial stiffness before glucose intolerance. *Intern Med.* 2014;53(3):189-94.

282. Davenport C, Ashley DT, O'Sullivan EP, Corley BT, Fitzgerald P, Agha A, et al. Identifying coronary artery disease in men with type 2 diabetes: osteoprotegerin, pulse wave velocity, and other biomarkers of cardiovascular risk. *J Hypertens*. 2011;29(12):2469-75.
283. Webb DR, Davies MJ, Gray LJ, Abrams KR, Srinivasan B, Das S, et al. Searching for the right outcome? A systematic review and meta-analysis of controlled trials using carotid intima-media thickness or pulse wave velocity to infer antiatherogenic properties of thiazolidinediones. *Diabetes Obes Metab*. 2010;12(2):124-32.
284. Davenport C, Ashley DT, O'Sullivan EP, McHenry CM, Agha A, Thompson CJ, et al. The Effects of Atorvastatin on Arterial Stiffness in Male Patients with Type 2 Diabetes. *Journal of diabetes research*. 2015;2015:846807.
285. Hagiwara K, Kuroki G, Yuan PX, Suzuki T, Murakami M, Hano T, et al. The effect of taurine on the salt-dependent blood pressure increase in the voltage-dependent calcium channel beta 3-subunit-deficient mouse. *J Cardiovasc Pharmacol*. 2003;41 Suppl 1:S127-31.
286. Mozaffari MS, Patel C, Abdelsayed R, Schaffer SW. Accelerated NaCl-induced hypertension in taurine-deficient rat: role of renal function. *Kidney Int*. 2006;70(2):329-37.
287. Yamori Y, Nara Y, Ikeda K, Mizushima S. Is taurine a preventive nutritional factor of cardiovascular diseases or just a biological marker of nutrition? *Adv Exp Med Biol*. 1996;403:623-9.
288. Liu F, Chen DD, Sun X, Xie HH, Yuan H, Jia W, et al. Hydrogen sulfide improves wound healing via restoration of endothelial progenitor cell functions and activation of angiotensin-1 in type 2 diabetes. *Diabetes*. 2014;63(5):1763-78.
289. Reinhard H, Jacobsen PK, Lajer M, Pedersen N, Billestrup N, Mandrup-Poulsen T, et al. Multifactorial treatment increases endothelial progenitor cells in patients with type 2 diabetes. *Diabetologia*. 2010.